

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

AKHVERDIAN et al.

Application No.: 10/673,786

Filing Date: September 30, 2003

For: METHOD FOR PRODUCING L-
THREONINE USING BACTERIA
BELONGING TO THE GENUS
ESCHERICHIA

Art Unit: 1652

Examiner: RAMIREZ, Delia M.

Attorney Ref. No.: US-115

Confirmation No.: 7880

BRIEF FOR APPELLANT

Mail Stop Appeal Brief - Patents

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

COMES NOW the Appellant to present this Brief in support of the appeal of the final rejections of Claims 12, 15-16, 19 and 21-24 in the above-captioned patent application. The Notice of Appeal having been timely filed on May 18, 2007, and a Petition with a one-month extension of time filed on May 18, 2007, this Brief is due to be filed on July 18, 2007.

It is not believed that extensions of time are required, beyond those that may otherwise be provided for in accompanying documents. If, however, additional extensions of time are necessary to prevent abandonment of this application or dismissal of this appeal, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and the Commissioner is hereby authorized to charge fees necessitated by this paper, and to credit all refunds and overpayments, to the credit card identified in EFS-Web processing.

For the following reasons, Appellant respectfully submits that the final rejection of each of Claims 12, 15-16, 19 and 23 in this application is in error, and therefore respectfully requests reversal of the rejections.

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I. Real Party in Interest

The real party in interest is Ajinomoto Co., Inc, a corporation of Japan.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of Claims

Claims 12, 15-16, 19 and 23 are pending. No claims are in condition for allowance. Claims 12, 15-16, 19 and 23 stand finally rejected in the Advisory Action dated May 11, 2007, and are on appeal.

IV. Status of Amendments

All amendments to the claims have been entered, including the amendment after final filed on April 18, 2007.

V. Summary of Claimed Subject Matter

The invention is directed a method for producing L- threonine comprising cultivating in a culture medium an L-threonine-producing *Escherichia coli* bacterium and collecting the L-threonine from the culture medium (see paragraph [0053]), wherein the bacterium has been modified to increase the expression of:

- i) the aspartate aminotransferase gene encoding the protein comprising the amino acid sequence of SEQ ID NO. 2 (see paragraph [0034]),
- ii) the *Escherichia coli thrA* gene which codes for an aspartokinase homoserine dehydrogenase I which is resistant to feedback inhibition by threonine (see paragraph [0051]),
- iii) the *Escherichia coli thrB* gene (see paragraph [0051]),
- iv) the *Escherichia coli thrC* gene (see paragraph [0051]), and
- v) the *Escherichia coli rhtA* gene (see paragraph [0052]),

wherein the expression of the genes is increased by a method selected from the group

consisting of increasing the copy number of said genes (see paragraph [0041]) and placing said genes under the control of a potent promoter (see paragraph [0043]).

VI. Ground of Rejection to Be Reviewed on Appeal

Whether Claims 12, 15-16, 19, and 23 are unpatentable under 35 U.S.C. §103 over the disclosure of Katsumata et al. in view of the disclosures of Debabov et al., Edwards et al., and further in view of Kishino et al..

VII. Argument

In the Final Rejection dated January 18, 2007, beginning at page 5, Claims 12, 15-16, 19, and 23 were rejected under 35 U.S.C. § 103 over Katsumata et al. in view of the disclosure of Debabov et al., Edwards et al., and further in view of Kishino et al. This rejection was upheld in the Advisory Action issued on May 11, 2007. For at least the following reasons, this rejection is in error and should be reversed.

A. Legal Standard

Claimed subject matter is obvious in light of the prior art if it would have been obvious to one of ordinary skill in the relevant art at the time the invention was made. 35 U.S.C. § 103(a). In considering the entire prior art in the relevant field, the claimed subject matter is obvious if the prior art "would have suggested to one of ordinary skill in the art that this [invention should be made] and would have a reasonable likelihood of success." *In re Dow Chemical Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988).

Obviousness can be shown either directly by demonstrating the technical motivation to combine the prior art, *Life Technologies, Inc. v. Clontech Laboratories, Inc.*, 224 F.3d 1320, 1326 (Fed. Cir. 2000), or indirectly through "secondary considerations" after the claimed subject matter was invented, *Custom Accessories, Inc. v. Jeffrey-Allan Industries, Inc.*, 807 F.2d 955, 960 (Fed. Cir. 1986). To show the motivation to combine prior art, it is not enough to simply identify different references that might be combined in hindsight; showing obviousness requires

showing a motivation to combine the pieces. *Velander v. Garner*, 348 F.3d 1359, 1363 (Fed. Cir. 2003). That motivation might come from a reference or from the knowledge of an artisan of ordinary skill. The level of ordinary skill in an art is based on a number of factors, including the educational level of the inventor, the type of problems encountered in the art, prior solutions to those problems, and the speed of innovation in the art. *Ruiz v. A.B. Chance Co.*, 234 F.3d 654, 666-67 (Fed. Cir. 2000).

“Secondary considerations” focus on how the invention was received in the market; a very successful or surprising invention is probably not obvious. *See Custom Accessories, Inc.*, 807 F.2d at 960. The “secondary considerations” considered by the courts include: commercial success, copying of the invention by others, licensing of the invention, evidence of a long-felt need for the invention, skepticism by skilled artisans that the claimed invention could be achieved, prior failures of others to achieve the same result, and unexpected results. *Id.*; *Pentec, Inc. v. Graphic Controls Corp.*, 776 F.2d 309, 316 (Fed. Cir. 1985). No secondary consideration is required for an invention to be non-obvious, but a court may use evidence of secondary considerations in its determination of obviousness (or non-obviousness). *Custom Accessories, Inc.*, 807 F.2d at 960.

Finally, the “teaching, suggestion, motivation” test (the “TSM” test) which is often used to prove obviousness was revised somewhat in a recent Supreme Court decision. *See KSR International Co. v. Teleflex Inc. et al.*, No. 04-1350, slip op. at 16 (S.Ct., April 30, 2007). This test, as applied in this case, was determined to be too rigidly applied. The Supreme Court said: “There is no necessary inconsistency between the idea underlying the TSM test and the Graham analysis. But when a court transforms the general principle into a rigid rule that limits the obviousness inquiry . . . it errs.” The Federal Circuit erred by (1) looking only at the problem the patentee was trying to solve, (2) assuming that a person of ordinary skill attempting to solve a problem will be led only to those elements of prior art designed to solve the same problem, (3) concluding that a patent claim cannot be proved obvious by showing the combination of elements was “obvious to try,” and (4) applying a rigid rule to prevent hindsight that denied factfinders “recourse to common sense.”

In its broadest sense, the *KSR* decision broadens the inquiry that must be made when proving an invention is obvious over prior art. Before this decision, when combining references, an Examiner must show a motivation by the person of skill in the art to combine the teachings of the different references. In the decision, the Supreme Court states that such a showing is not required, but only a “reason” to combine the teachings should be shown. This reason can be based upon logic, common sense, and/or the knowledge in the art. The Court stated that when familiar elements are combined according to known methods, the invention is likely to be obvious when it does no more than yield predictable results.” *KSR, slip op.* at 12.

Also, the Court explained that when the prior art elements work together in an unexpected and fruitful manner, the invention is not obvious. *Id.* (citing *United States v. Adams*, 383 U.S. 39, 40 (1966)). If, however, the combination of old elements does no more than they would in separate, sequential operation, even though the combination might perform a useful function, the combination is likely obvious. *Id.* at 13 (citing *Anderson’s-Black Rock, Inc. v. Pavement Salvage Co.*, 396 U.S. 57 (1969)).

These statements regarding expectation are not so different from the current test for proving obviousness, however, the Court does broaden the analysis in its discussion of expanding of the “teaching, suggestion, or motivation” test. As stated above, a specific or rigid motivation is no longer required, but only a logical reason for combining prior art. This standard is less stringent.

B. The rejection of Claims 12, 15-16, 19 and 23 under 35 U.S.C. §103 is in error

In the January 18, 2007 Final Office Action, claims 12, 15-16, 19, and 23 were rejected under 35 U.S.C. §103 over Katsumata et al. in view of the disclosures of Debabov et al., Edwards et al., and further in view of Kishino et al..

The present invention relates to a method for producing L-threonine comprising cultivating an L-threonine producing *E. coli* which has been modified to enhance the activity of aspartate aminotransferase, otherwise known as AspC, having the amino acid sequence of SEQ ID NO: 2, as well as enhancing the expression of the following known genes: a feedback-

resistant *thrA*, *thrB*, *thrC*, and *rhtA*. All of the genes are derived from *Escherichia coli*.

Many types of aminotransferase enzymes are known, and their substrate specificities are each different. For example, it is described in the left column of the page 7639, J. Bacteriol., 187, 7639-7646 (2005) [EXHIBIT A, submitted with the response filed October 19, 2006 and attached hereto], that 16 types of aminotransferases exist in *E. coli*, and 14 types in *Corynebacterium glutamicum*. These aminotransferases are involved in the synthesis of many kinds of amino acids. It is not clear from the prior art, however, which type of aminotransferase is effective for increasing a production of L-threonine.

Katsumata et al. disclose L-threonine production using an aminotransferase gene cloned from *Corynebacterium glutamicum*; however, it is clear that the disclosed gene is not the aspartate aminotransferase depicted in SEQ ID NO. 2. In fact, it is not clear what type of aminotransferase is encoded by the prior art gene. As the Office Action acknowledges, Katsumata et al. does not teach production of L-threonine, nor the aspartate aminotransferase of SEQ ID NO. 2.

Debabov et al., Edwards et al., and Kishino et al. fail to make up for the deficiencies of Katsumata et al.. Debabov et al. also fails to teach the aspartate aminotransferase of SEQ ID NO. 2, but teach increased expression of *thrA*, *thrB*, *thrC*, and *rhtA*. Since there is no disclosure of the aspartate aminotransferase of SEQ ID NO. 2, this reference fails to make up for the deficiency of Katsumata et al.

Edwards et al. is cited for disclosing that aspartate aminotransferase is effective for L-phenylalanine production in *E. coli*. However, the L-threonine biosynthesis pathway is completely different from the L-phenylalanine biosynthesis pathway. Namely, L-phenylalanine is generated from phenyl pyruvate acid by transamination catalyzed by aminotransferase, whereas L-threonine is not generated by direct transamination catalyzed by aminotransferase. From this teaching, one of ordinary skill in the art would not have been able to determine or deduce that increasing the expression of the aspartate aminotransferase gene of *E. coli* would be effective for producing L-threonine, since the production pathway of L-phenylalanine is completely different from that of L-threonine. Therefore, Edwards et al. fails to make up for the

deficiencies of Katsumata et al..

Kishino et al. teach the use of low copy vectors in preferred strains of *E. coli* for L-threonine production; however, they fail to teach the method of increasing expression of the gene of SEQ ID NO. 1 to increase L-threonine production. Therefore, Kishino et al. fail to make up for the deficiencies of Katsumata et al..

Neither the primary reference of Katsumata et al., nor any of the secondary references, teach L-threonine production in *E. coli* which have been modified to have increased gene expression of the aspartate aminotransferase gene, depicted in SEQ ID NO. 1 and encoding a protein depicted in SEQ ID NO. 2. Furthermore, no combination of the teachings would suggest such a teaching. As the main feature of the claimed invention, the connection between the increase in gene expression of the gene encoding aspartate aminotransferase of SEQ ID NO.2 and the increased production of L-threonine, is not remotely suggested by the cited references, either singly or in any combination.

On page 6 of the Office Action issued January 18, 2007, it is argued that Katsumata teach a protein having the same enzymatic activity as the polypeptide of SEQ ID NO: 2, and the increase in L-threonine production as a result of increasing the synthesis of an enzyme from *C. glutamicum* having aspartate aminotransferase activity. Furthermore, it is argued that Debabov et al. is a high L-threonine producer which has been modified to increase expression of the *thrA*, *thrB*, *thrC*, and *rhtA* genes.

It would not have been expected by the ordinarily skilled art worker that an additional increase in threonine production would result by increasing the expression of the gene depicted in SEQ ID NO: 2, because threonine production is *already* optimized at a high level in a bacterium with increased expression of the *thrA*, *thrB*, *thrC*, and *rhtA* genes. Furthermore, it is acknowledged (and pointed out in the January 18, 2007 Office Action on page 6) that the *aspC* gene (SEQ ID NO: 2) encodes an enzyme which catalyzes the synthesis of aspartic acid from oxaloacetic acid. However, one of ordinary skill in the art would know that even if expression of the *aspC* gene is increased, the production of threonine would not also increase unless the supply of aspartic acid runs short in the whole pathway of threonine synthesis. The shortage of aspartic

acid will occur if the synthesis reaction of aspartic acid from oxaloacetic acid is the rate-limiting step. However, one of ordinary skill in the art would not have known which reaction is the rate-limiting step in threonine synthesis. Therefore, the additional increase in threonine as a result of the combination of increasing expression of the *aspC* gene with increasing expression of the *thrA*, *thrB*, *thrC* and *rhtA* genes is completely unexpected.

Thus, one of ordinary skill in the art would know that the achieving increased threonine production will not occur merely by increasing the expression of *aspC* in *E. coli* which has increased expression of *thrA*, *thrB*, *thrC*, and *rhtA*. Therefore, the result of the present claimed method is completely unexpected, and is a novel and unobvious result.

Turning to the Advisory Action dated May 11, 2007, it is stated on page 2 that “[t]here is no evidence in the specification or the art that the maximum amount of L-threonine that can be produced in *E. coli* is that obtained from an *E. coli* which has increased expression of the *thrA*, *thrB*, *thrC*, and *rhtA* genes”. First, the claims do not require the ‘maximum amount’ of L-threonine to be produced. Furthermore, the specification clearly describes in paragraph [0005] the threonine producing strain B-3996, which is known to be the best threonine producer at the time of filing of the instant application, and which has enhanced expression of the *thrA*, *thrB*, *thrC*, and *rhtA* genes.

On page 3-4 of the Advisory Action, it is stated that “it is clear from the art that there is not only motivation but a reasonable expectation of success at increasing production of L-threonine by increasing the expression of the *aspC* gene.” The Action further references paragraph [0010] of the specification and the art cited therein as teaching “that a similar approach (increased expression of the *aspC* gene) was followed in the production of L-lysine in *E. coli*, which is also synthesized from aspartic acid, that resulted in increased production of L-lysine”. This assessment is simply incorrect. The art cited in the specification which is referred to in the Advisory Action (US Patent 6,040,160 – provided as Exhibit B as evidence relied upon by the Examiner in any ground of rejection to be reviewed upon appeal, 37 C.F.R.

41.67(c)(1)(ix)) contains tables 8, 10, 12, 14, 18, 19, 20, and 21, which show the effect of increasing expression of various genes on the production of L-lysine. In these tables, “*paspC*”

indicates a plasmid containing the aspC gene. It can be seen from these tables that the production of L-lysine is hardly increased in all strains transformed with the paspC. It is clear from these data that increasing the expression of the aspC gene does not lead to increased L-lysine production in a strain wherein the supply of aspartic acid is not rate-limiting for L-lysine production. Similarly, in the production of threonine in *E.coli*, increasing the expression of the aspC gene does not lead to increased threonine production unless the supply of aspartic acid is rate-limiting. Therefore, since the threonine synthetic pathway is very complex and the rate-limiting step is unknown, it would not have been obvious to one of ordinary skill in the art that increasing the aspC expression would lead to increased threonine production. The present invention is the first to show that threonine production is increased by combining increased expression of the aspC, thrA, thrB, thrC, and rhtA genes all together, which is clearly not expected by the combination of the cited prior art.

For at least the foregoing reasons, Appellants respectfully submit that the subject matters of the Claims, each taken as a whole, would not have been obvious to one of ordinary skill in the art at the time of Appellant's invention, are therefore not unpatentable under 35 U.S.C. § 103(a), and therefore respectfully request withdrawal of the rejection thereof under 35 U.S.C. § 103(a).

IX. Conclusion

For at least the foregoing reasons, Appellant respectfully submits that the subject matters of Claims 12, 15-16, 19, and 23, each taken as a whole, are patentable. Accordingly, Appellant respectfully requests reversal of the rejections of Claims 12, 15-16, 19, and 23 under section 103.

Respectfully submitted,

By: 

Shelly Guest Cermak
Registration No. 39,571

U.S. P.T.O. Customer Number 38108
Cermak Kenealy & Vaidya, LLP
515-B E. Braddock Road
Alexandria, VA 22314
703.778.6608 (v)
703.652.5101 (f)

Date: July 18, 2007

APPENDIX A: CLAIMS ON APPEAL

12. A method for producing L- threonine comprising:

A) cultivating in a culture medium an L-threonine-producing *Escherichia coli* bacterium, wherein the bacterium has been modified to increase the expression of:

i) the aspartate aminotransferase gene encoding the protein comprising the amino acid sequence of SEQ ID NO. 2,

ii) the *Escherichia coli thrA* gene which codes for an aspartokinase homoserine dehydrogenase I which is resistant to feedback inhibition by threonine,

iii) the *Escherichia coli thrB* gene,

iv) the *Escherichia coli thrC* gene, and

v) the *Escherichia coli rhtA* gene,

wherein said expression of said genes is increased by a method selected from the group consisting of increasing the copy number of said gene and placing said gene under the control of a potent promoter, and

B) collecting the L-threonine from the culture medium.

15. The method according to claim 12, wherein said expression of the aspartate aminotransferase gene is increased by increasing the copy number of the aspartate aminotransferase gene.

16. The method of claim 15, wherein the copy number is increased by transforming said bacterium with a low copy number vector containing said gene.

19. The method of claim 12, wherein said aspartate aminotransferase gene comprises a DNA comprising the nucleotides 1 to 1191 in SEQ ID NO: 1.

23. The method according to claim 12, wherein the potent promoter is selected from the

group consisting of the lac promoter, trp promoter, trc promoter, PR promoter, and PL promoter.

APPENDIX B: EVIDENCE

Exhibit A: Marienhagen, et al., J. Bacteriol., 187, 7639-7646 (2005), particularly page 7639, submitted with the response filed October 19, 2006.

Exhibit B: US Patent 6,040,160 – provided as evidence relied upon by the Examiner in any ground of rejection to be reviewed upon appeal, see 37 C.F.R. 41.67(c)(1)(ix), cited to by the Examiner in the Advisory Action dated May 11, 2007.

APPENDIX C: RELATED PROCEEDINGS

None.

EXHIBIT A

Functional Analysis of All Aminotransferase Proteins Inferred from the Genome Sequence of *Corynebacterium glutamicum*

Jan Marienhagen, Nicole Kennerknecht, Hermann Sahm, and Lothar Eggeling*

Institute of Biotechnology, Research Centre Juelich, D-52425 Juelich, Germany

Received 3 June 2005/Accepted 8 August 2005

Twenty putative aminotransferase (AT) proteins of *Corynebacterium glutamicum*, or rather pyridoxal-5'-phosphate (PLP)-dependent enzymes, were isolated and assayed among others with L-glutamate, L-aspartate, and L-alanine as amino donors and a number of 2-oxo-acids as amino acceptors. One outstanding AT identified is AlaT, which has a broad amino donor specificity utilizing (in the order of preference) L-glutamate > 2-aminobutyrate > L-aspartate with pyruvate as acceptor. Another AT is AvtA, which utilizes L-alanine to aminate 2-oxo-isovalerate, the L-valine precursor, and 2-oxo-butyrate. A second AT active with the L-valine precursor and that of the other two branched-chain amino acids, too, is IlvE, and both enzyme activities overlap partially in vivo, as demonstrated by the analysis of deletion mutants. Also identified was AroT, the aromatic AT, and this and IlvE were shown to have comparable activities with phenylpyruvate, thus demonstrating the relevance of both ATs for L-phenylalanine synthesis. We also assessed the activity of two PLP-containing cysteine desulfurases, supplying a persulfide intermediate. One of them is SufS, which assists in the sulfur transfer pathway for the Fe-S cluster assembly. Together with the identification of further ATs and the additional analysis of deletion mutants, this results in an overview of the ATs within an organism that may not have been achieved thus far.

As inferred from the genome sequences, bacteria possess a number of aminotransferase (AT) proteins which, according to the KEGG entries, amount in the proteobacterium *Escherichia coli* to at least 16 and in the actinobacterium *Corynebacterium glutamicum* to 14 different proteins. The majority of these proteins are involved in amino acid synthesis, or amino acid interconversion, but also in the synthesis of biotin and porphyrin (10, 12). All ATs are pyridoxal-5'-phosphate (PLP)-dependent enzymes, where PLP forms the aldimine intermediate during transfer of the amino group from the incoming amino acid to an α -keto acid forming a new amino acid (20). However, the PLP-aldimine intermediate enables a wide variety of further reactions such as, for instance, C-S lyase activity by α,β -elimination or decarboxylation (13). Due to the mechanistic similarity of PLP-catalyzed reactions, the large number of AT proteins present, and their closely related structure, it is usually difficult, if not impossible, to derive the function of these proteins solely based on sequence studies.

An additional distinct feature of the ATs is their overlapping substrate specificity, which often leads to the nonexistence of a phenotype if one of them is absent. Thus, in *E. coli* the three ATs encoded by *tyrB*, *aspC*, and *ilvE* are involved in the synthesis of the aromatic amino acids, and the individual in vivo contribution of each of these ATs could only be studied when the other two respective genes were inactivated (7). Another example is the contribution of *dapC* and *argD* to L-lysine synthesis in *E. coli* (16) and *C. glutamicum* as well, with the latter organism possibly even possessing a third activity (9).

As mentioned above, a large number of bacterial ATs are involved in amino acid synthesis, and it is clear that these

proteins are of special relevance for amino acid production with *C. glutamicum*. Functionally identified ATs of this organism include the *N*-succinyl-2,6-diaminopimelate AT (*dapC*) involved in the synthesis of L-lysine, of which 650,000 tonnes per year (t/y) are produced with *C. glutamicum* (5), as well as *ilvE*, encoding the branched-chain AT necessary for L-isoleucine production (26). Mutant studies further identified the genes (and respective enzymes) *bioA* (adenosylmethionine-8-amino-7-oxononanoate aminotransferase) (10), *argD* (*N*-acetyl-ornithine AT) (29), and *pat* and *pdxR* (involved in aromatic amino acid and pyridoxal-5'-phosphate synthesis, respectively) (19). Furthermore, an activity has been identified that uses L-alanine as the amino donor (17), thus resembling AvtA of *E. coli* (37). The function of some of these ATs was derived from a recent bioinformatic approach identifying a total of 20 sequences with similarities to ATs in the genome of *C. glutamicum* (19). However, bioinformatic and mutant analyses failed to identify specific ATs such as, for instance, the corresponding counterparts to *avtA*, *aspC*, or *tyrB* of *E. coli*.

Based on the recent bioinformatic study, we here isolate the AT proteins of *C. glutamicum* to study their activity with a variety of substrates. Together with in vivo studies, this investigation is an attempt to make a functional assignment of the ATs known from the genome analysis of a bacterium.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. The standard medium for *E. coli* was Luria broth. *C. glutamicum* was precultivated on brain heart infusion (Difco) with subsequent cultivation on the minimal medium CGXII (5). When appropriate, chloramphenicol (25 mg liter⁻¹) or kanamycin (15, 25, or 50 mg liter⁻¹) was added to the medium. *E. coli* was grown at 30 or 37°C, and *C. glutamicum* was grown at 30°C.

Construction of plasmids. Plasmids were constructed in *E. coli* DH5 α MCR from PCR-generated fragments (Expand High Fidelity PCR kit: Roche Diagnostics) by using *C. glutamicum* ATCC 13032 DNA as a template. In order to construct pJM Δ ilvE the upstream primer 5'-ATGGATGGTCTCAAATGATTC

* Corresponding author. Mailing address: Institute of Biotechnology, Research Centre Juelich, D-52425 Juelich, Germany. Phone: 49-2461-61-5132. Fax: 49-2461-612710. E-mail: l.eggeling@fz-juelich.de.

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics ^a	Reference or source ^b
Strains		
<i>E. coli</i> DH5 α MCR	F ⁻ <i>endA1 supE44 thi-1 λ-recA1 gyrA96 relA1 deoR Δ(lacZYA-argF)U169 ϕ80dlacZAM15 mcrA Δ(mrr-hsdRMS-mcrBC)</i>	8
<i>C. glutamicum</i>		
ATCC 13032	WT	ATCC
WT Δ ilvE	WT deleted of a 1,050-nt fragment of <i>ilvE</i>	This study
WT Δ alaT	WT deleted of a 1,260-nt fragment of <i>alaT</i>	This study
WT Δ aroT	WT deleted of a 972-nt fragment of <i>aroT</i>	This study
WT Δ avtA	WT deleted of a 1,107-nt fragment of <i>avtA</i>	This study
WT Δ alaT Δ ilvE	WT deleted of a 1,260-nt fragment of <i>alaT</i> and a 1,050-nt fragment of <i>ilvE</i>	This study
WT Δ aroT Δ ilvE	WT deleted of a 972-nt fragment of <i>aroT</i> and a 1,050-nt fragment of <i>ilvE</i>	This study
WT Δ avtA Δ ilvE	WT deleted of a 1,107-nt fragment of <i>avtA</i> and a 1,050-nt fragment of <i>ilvE</i>	This study
Plasmids		
pASK-IBA-3C	Vector for heterologous gene expression in <i>E. coli</i> Cm ^r <i>oriV_{EC}</i> <i>tetR</i>	33
pJMargD	pASK-IBA-3C with <i>argD</i> (1467379–1467398, 1468548–1468527)	This study
pJMilvE	pASK-IBA-3C with <i>ilvE</i> (2337049–2337028, 2335916–2335935)	This study
pJMavtA	pASK-IBA-3C with <i>avtA</i> (2766133–2766111, 2764979–2765003)	This study
pJMalaT	pASK-IBA-3C with <i>alaT</i> (3030673–3030694, 3031980–3031959)	This study
pJM0780	pASK-IBA-3C with the coding sequence of NCgl0780 (861547–861569, 862752–862730)	This study
pJMaroT	pASK-IBA-3C with the coding sequence of NCgl2355 (2584563–2584584, 2585927–2585909)	This study
pJMhisC	pASK-IBA-3C with <i>aroT</i> (233279–233256, 232260–232279)	This study
pJMhemI	pASK-IBA-3C (2217588–2217565, 2216494–2216515)	This study
pJM2355	pASK-IBA-3C with <i>hemI</i> (462560–462581, 463867–463847)	This study
pJM2491	pASK-IBA-3C with the coding sequence of NCgl2355 (2584563–2584584, 2585927–2585909)	This study
pJMstfS	pASK-IBA-3C with the coding sequence of NCgl2491 (2742575–2742553, 2741637–2741657)	This study
pJMserC	pASK-IBA-3C (1649407–1649388, 1648100–1648122)	This study
pJMbioA	pASK-IBA-3C with <i>serC</i> (877121–877101, 875985–876005)	This study
pJMaspT	pASK-IBA-3C with <i>bioA</i> (2770718–2770737, 2771983–2771961)	This study
pJMaccD	pASK-IBA-3C with <i>aspT</i> (256620–256641, 257894–257874)	This study
pJMdapC	pASK-IBA-3C with <i>accD</i> (2444610–2444632, 2445710–2445691)	This study
pJMpdxR	pASK-IBA-3C with <i>dapC</i> (1149282–1149300, 1150379–1150359)	This study
pJM0462	pASK-IBA-3C with <i>pdxR</i> (830982–830963, 829627–829646)	This study
pJM1184	pASK-IBA-3C with the coding sequence of NCgl0462 (501499–501518, 502920–502901)	This study
pJM1022	pASK-IBA-3C with the coding sequence of NCgl1184 (1297215–1297238, 1298339–1298319)	This study
pK19mobsacB	pASK-IBA-3C with the coding sequence of NCgl1022 (1116902–1116881, 1115832–1115851)	This study
pK19mobsacB Δ ilvE	Integration vector: Km ^r <i>oriV_{EC}</i> <i>oriT</i> <i>sacB</i>	31
pK19mobsacB Δ alaT	Plasmid to delete a 1,050-nt fragment of the <i>C. glutamicum</i> chromosome (2336998–2335949)	This study
pK19mobsacB Δ aroT	Plasmid to delete a 1,260-nt fragment of the <i>C. glutamicum</i> chromosome (3030688–3031947)	This study
pK19mobsacB Δ avtA	Plasmid to delete a 972-nt fragment of the <i>C. glutamicum</i> chromosome (233264–2332293)	This study
pK19mobsacB Δ ilvE	Plasmid to delete a 1,107-nt fragment of the <i>C. glutamicum</i> chromosome (2766118–2765012)	This study

^a Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance. Subscripts: *Ec*, *E. coli*. The nucleotide numbers of the expression vectors refer to the genome sequence BA000036 and correspond to the specific part present in the primer used to amplify the gene. The numbers of the four deletion vectors at the bottom of the table refer to the nucleotides that are deleted in the chromosome upon use of these vectors. For details, see the text.

^b ATCC, American Type Culture Collection.

TGTCAGGATGTCAGGTGAT-3' was used. The underlined sequence is specific for *ilvE* and corresponds to nucleotides (nt) 2337049 to 2337028 of the *C. glutamicum* genome sequence BA000036. As the downstream primer, 5'-ATG GATGGTCTCAGCGCTGCCAACCAGTGGGATAAGCC-3' was used, with the underlined sequence corresponding to nt 2335916 to 2335935. In Table 1 only the gene-specific nt numbers are given for the primers to amplify the respective Δ Ts. The sequences common to all primers used for gene amplification are identical to those given in boldface for *ilvE*. The resulting fragments were BsaI digested and cloned into the BsaI site of pASK-IBA-3C (IBA GmbH, Göttingen, Germany). Accordingly, the open reading frames of the other 19 genes encoding potential ATs were cloned into pASK-IBA-3C. To enable chromosomal deletions of *ilvE*, *alaT*, *aroT*, and *avtA*, crossover PCR was applied (18) to generate a defined fragment of approximately 875 bp in size carrying upstream and downstream sequences of about equal size of the respective open reading frame to be deleted. The fragments were cloned into pK19mobsacB via their attached BamHI sites. The plasmids made eventually enabled a defined chromosomal deletion, as specified by the nucleotide numbers of the wild-type (WT) chromosome (Table 1).

Construction of strains. *C. glutamicum* was transformed by electroporation (34). The Δ T deletion mutants were constructed by using pK19mobsacB Δ ilvE,

pK19mobsacB Δ alaT, pK19mobsacB Δ aroT, and pK19mobsacB Δ avtA, respectively. Clones were selected for kanamycin resistance to establish integration of the plasmid in the chromosome. In a second round of positive selection by using sucrose resistance, clones were selected for deletion of the vector (31). The deletions in the chromosome were verified by PCR analysis using primers hybridizing approximately 500 bp upstream and 500 bp downstream of the open reading frames in question.

Heterologous gene expression and protein purification. The 20 *E. coli* DH5 α MCR strains, each one harboring a different pASK-IBA-3C derivative encoding a potential AT were grown until the optical density at 550 nm reached 0.5. After induction by adding 10 μ l of anhydrotetracycline (2 mg ml⁻¹), the cultures were incubated for 3 h at 30°C. The cells were harvested by centrifugation at 6,000 rpm for 12 min at 4°C. Crude extracts were obtained by sonification using a Branson Sonifier 250 (intensity, 2; duty cycle, 20%; 4 min; Branson, Danbury, CT), while cooling on ice. After removal of the cellular debris by centrifugation (15 min, 14,000 rpm, 4°C), all preparation procedures were performed at 4°C using *Strep*-Tactin Sepharose and the *Strep*-tag Protein Purification Buffer Set (IBA GmbH, Göttingen, Germany). Purified proteins were stored in the elution buffer (100 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA) at -20°C. The identity of the potential AT proteins were confirmed by using matrix-assisted

laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). Protein concentrations were determined by using a BCA Protein Assay Kit (Pierce, Rockford, IL).

MALDI-TOF-MS. For MALDI-TOF-MS, protein spots stained with Coomassie brilliant blue were excised from the sodium dodecyl sulfate gel and washed three times with 750 μ l of 30% CH_3CN -100 mM NH_4HCO_3 . After drying the gel pieces and reswelling them in 12 μ l of 3 mM NH_4HCO_3 containing 5 ng of modified trypsin (Promega, Mannheim, Germany), digestion was carried out overnight at 37°C. Elution of the peptides from the gel was performed by the addition of 10 μ l of 30% CH_3CN -0.1% trifluoroacetic acid. For MALDI-TOF-MS, 2 μ l of each supernatant was mixed with 0.5 μ l of saturated α -cyano-4-hydroxycinnamic acid matrix (20 mg ml⁻¹) prepared in 0.25% trifluoroacetic acid-50% acetonitrile. This mixture was spotted onto the sample probe, and MALDI mass spectra were obtained with a PerSeptive Biosystems Voyager DE STR mass spectrometer (PerSeptive Biosystems, Langen, Germany). For calibration of the mass spectrometer, the Sequazyme peptide mass standard kit (Applied Bioscience, Wieterstadt, Germany) was used. Monoisotopic masses were assigned and used for in-house database searches of the *C. glutamicum* genome performed with the Gpmaw software (Lighthouse Data, Odense, Denmark).

Crude extracts. *C. glutamicum* was grown in minimal medium until the optical density at 600 nm reached 10 (exponential phase). The cells were harvested by centrifugation for 15 min at 4,000 rpm at 4°C. All preparation procedures were performed at 4°C. The pellet was washed twice with 200 mM Tris-HCl (pH 8) and was resuspended in the same buffer. Crude extracts were obtained by sonication, and after centrifugation (15 min, 14,000 rpm, 4°C) the supernatant was desalted with PD-10 columns (Pharmacia, Uppsala, Sweden) and kept on ice until enzyme assays were performed.

Enzyme assays. The AT assay contained 200 mM Tris-HCl (pH 8), 0.25 mM pyridoxal-5'-phosphate, 4 mM keto acid, and 50 mM L-amino acid. The reaction was started by the addition of purified protein or crude extract (in 1 ml) and was performed at 30°C. Several 50- μ l samples were collected over a period of 20 min. The reaction was terminated by mixing each sample with 30 μ l of 5% perchloric acid and 38% ethanol. After the sample was neutralized by the addition of 20 μ l of 20 mM Tris-HCl (pH 8) buffer with 23 mM K_2CO_3 , the precipitated salts were removed by centrifugation (10 min, 13,000 rpm). Subsequently, amino acids were quantified by high-pressure liquid chromatography as their *o*-phthaldehyde derivative. Assays were linear over time and proportional to the protein concentration used.

The cysteine desulfurase assays were performed with 50 mM L-cysteine and 2.5 mM pyridoxal-5'-phosphate in 20 mM Tris-HCl (pH 8). The reaction mixture was incubated at ambient temperature for 2.5 h, and samples taken at different points in time to quantify the L-alanine formed by high-pressure liquid chromatography.

RESULTS

Isolation of AT proteins and activity tests. Application of hidden Markov models identified 20 genes in the genome of *C. glutamicum* putatively encoding ATs (19). We cloned all of these genes into pASK-IBA-3C, expressed them in *E. coli*, and isolated the proteins fused at their carboxy-terminal ends with Strep-tag II via affinity purification. In each case, 0.6 to 2.4 mg of protein was obtained from a 100-ml culture. The protein was pure as judged by SDS-PAGE analysis. Its identity was confirmed by MALDI-TOF-MS.

Due to our interest in L-isoleucine synthesis with *C. glutamicum* (22), we first focused on branched-chain amino acid synthesis. All 20 proteins were individually assayed with amino donor L-Glu, L-Ala, L-Asp, or L-Gln using as the amino acceptor L-2-oxo-3-methylvalerate (O-Ile), 2-oxo-isocaproate (O-Leu), or 2-oxo-isovalerate (O-Val). In addition, the L-Ile intermediate 2-oxo-butyrate (O-But) was assayed, which is known to be formed during L-Ile production (38). Product formation was followed over time, and the results where detectable amino acid formation occurred are shown in Table 2. In order to avoid confusion in the nomenclature, the enzyme names resulting in the course of the studies are already given in this table. Of the 20 proteins, 5 exhibited AT activity with O-Ile,

TABLE 2. Activities of the AT proteins of *C. glutamicum* with detectable activities toward branched-chain amino acid intermediates or 2-oxo-butyrate

AT ^a	Amino donor ^b	Amino acceptor	Sp act ^c ($\mu\text{mol min}^{-1}\text{ mg}$ of protein ⁻¹)
ArgD	L-Glu	O-But	0.1
	L-Ala	O-But	0.1
	L-Asp	O-But	0.1
	L-Gln	O-But	0.1
IleE	L-Glu	O-Ile	9.6
	L-Glu	O-Leu	13.9
	L-Glu	O-Val	13.7
	L-Glu	O-But	4.3
AvlA	L-Ala	O-Ile	3.7
	L-Ala	O-Leu	0.9
	L-Ala	O-Val	18.2
	L-Ala	O-But	27.5
	L-Gln	O-Ile	0.1
	L-Gln	O-Leu	0.1
	L-Gln	O-Val	0.1
	L-Gln	O-But	0.1
AlaT	L-Glu	O-But	5.4
	L-Ala	O-But	3.0
	L-Asp	O-But	2.3
	L-Gln	O-But	0.7
NCgl0780	L-Glu	O-Leu	0.1
	L-Glu	O-But	0.2
AroT	L-Glu	O-Leu	1.3
	L-Ala	O-Leu	0.8
	L-Asp	O-Leu	0.1
	L-Gln	O-Leu	0.1
	L-Glu	O-Val	0.1
	L-Ala	O-Val	0.1
	L-Asp	O-Val	0.1
	L-Gln	O-Val	0.1
	L-Glu	O-But	1.1
	L-Ala	O-But	0.7
	L-Asp	O-But	0.1
	L-Gln	O-But	0.1
HisC	L-Glu	O-Leu	0.8
	L-Ala	O-Leu	0.1
	L-Asp	O-Leu	0.4
	L-Gln	O-Leu	0.1
	L-Glu	O-But	0.2
	L-Ala	O-But	0.1
NCgl0462	L-Asp	O-But	0.1
	L-Gln	O-But	0.1
	L-Glu	O-But	0.1
	L-Ala	O-But	0.1

^a Either names or NCgl numbers are given. See also Table 4.

^b Amino donors are given as their three-letter code; amino acceptors are as follows: O-Ile, 2-oxo-3-methylvalerate; O-Leu, 2-oxo-isocaproate; O-Val, 2-oxo-isovalerate; and O-But, 2-oxo butyrate.

^c 0.1 means detectable amino acid formation compared to the other 12 ATs not included in the table. Each value represents the average of at least two independent assays.

O-Leu, O-Val, and O-But, and 3 additional AT proteins had weak activities with O-But only but not with the ultimate branched-chain amino acid intermediates. With the other 12 proteins no activities were found in these assays. The highest

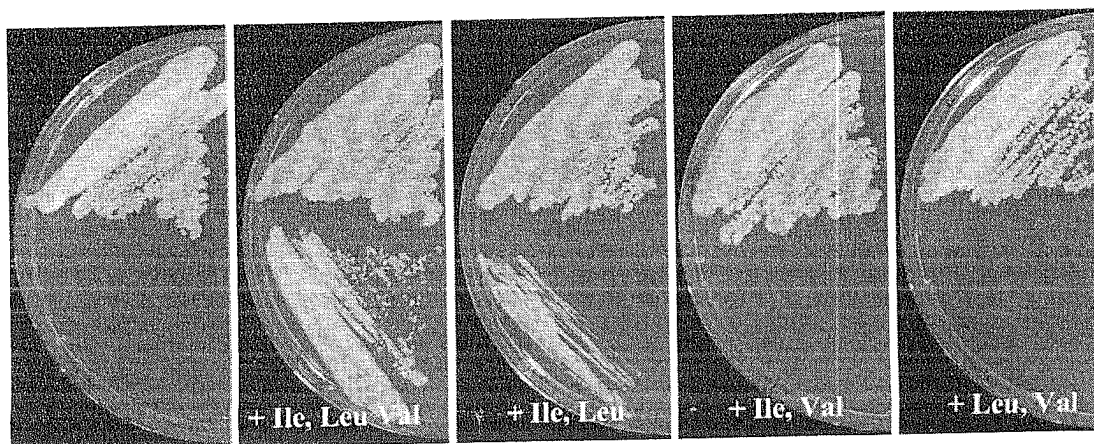


FIG. 1. Branched-chain amino acid requirements of *C. glutamicum* $\Delta ilvE$. At the top is shown the WT, and the deletion mutant is shown at the bottom. Growth was carried out on salt medium CGXII (5) with amino acids supplemented as indicated (each at 1 mM).

activities were not only present for IlvE, which had been previously identified (26), but also for the AT termed AvtA. The latter AT uses L-Ala as the amino donor instead of L-Glu and thus resembles transaminase C of *E. coli* (37), and a corresponding activity of *C. glutamicum* was recently described (17).

IlvE and AvtA affinities. As mentioned, the identified proteins, IlvE and AvtA, have the highest specific activities with the substrates assayed, but they use different amino donors. The *ilvE* gene was originally isolated by complementation of a mutant of *C. glutamicum* requiring all three branched-chain amino acids (26), and therefore an *in vivo* function of AvtA is not directly apparent. We therefore determined the substrate affinities for both proteins in Lineweaver-Burk plots (not shown). With the amino donor L-Glu the K_m (mM) for IlvE was 0.23 (O-Ile), 0.15 (O-Leu), 0.63 (O-Val), and 1.42 (O-But). For AvtA with L-Ala as amino donor the K_m values were 3.52 (O-Ile), 16.84 (O-Leu), 2.51 (O-Val), and 0.60 (O-But). This agrees with the view that the major function of IlvE is synthesis of the branched-chain amino acids and that O-But amination probably represents a side activity of this protein. Compared to this, AvtA has weak affinities for the branched-chain amino acid intermediates. The highest affinity and also activity (Table 2) was present for O-But, an activity that may not be considered to represent a housekeeping function. No activity of AvtA was detected with the substrates glycine and pyruvate.

***In vivo* IlvE and AvtA function.** In order to analyze *in vivo* branched-chain amino acid synthesis, we deleted *avtA* and *ilvE* in the chromosome of the WT of *C. glutamicum* ATCC 13032, singly or combined, and assayed growth on mineral salts medium. WT $\Delta ilvE$ was fully dependent on L-Ile and L-Leu supply but not on the supply of L-Val (Fig. 1). In contrast, WT $\Delta avtA$ did not exhibit a phenotype on CGXII (not shown). This illustrates that *in vivo*, at least for L-Ile and L-Leu synthesis, IlvE is clearly the major AT activity. The situation with L-Val is different. WT $\Delta ilvE$ still exhibits significant growth without L-Val addition, which is disabled in WT $\Delta ilvE \Delta avtA$ (Fig. 2). Therefore, AvtA contributes to L-Val synthesis *in vivo*, and the *avtA* deletion is silent unless *ilvE* is deleted as is similarly the case for *E. coli* (2). We did growth experiments with strain WT $\Delta avtA$

to pursue the idea that AvtA might actually be necessary to catabolize externally supplied L-Ala, performed on complex medium brain heart infusion, as well as on salt medium CGXII with or without 20 mM L-Ala, but these investigations were without observable effects.

As a further characterization of the *in vivo* function, activities in crude extracts of the WT and the mutants grown on CGXII were compared (Table 3). From the comparison of WT with WT $\Delta avtA$ we conclude that (i) AvtA has highest activities with O-Val and O-But as has the isolated protein (Table 2), (ii) there is no further Ala-dependent activity for O-Val formation, and (iii) there are further Ala-dependent activities for O-But

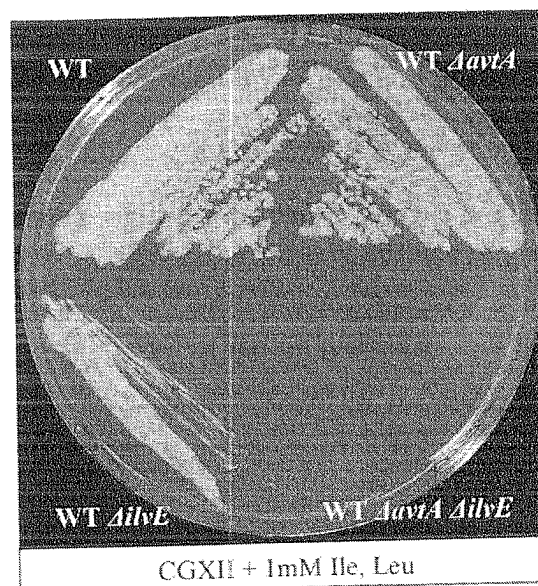


FIG. 2. L-Val synthesis by AvtA. The salt medium CGXII was supplemented with L-Ile plus L-Leu. The isogenic mutants, derived from *C. glutamicum* WT, are as indicated.

TABLE 3. Specific activities in crude extracts of mutants^a

Amino donor	Amino acceptor	Sp act ($\mu\text{mol min}^{-1}$ mg of protein ⁻¹)		
		WT	WT ΔalaT	WT ΔilvE
L-Glu	O-Ile	38	35	<1
	O-Leu	44	45	<1
	O-Val	24	21	<1
	O-But	61	55	18
L-Ala	O-Ile	5	<1	3
	O-Leu	<1	<1	<1
	O-Val	19	<1	16
	O-But	49	35	26

^a Measurements were done twice with variations of <12%.

formation. In WT ΔilvE all Glu-dependent formation of branched-chain amino acids is absent, but there is still Glu-dependent formation of aminobutyrate, which agrees with the large number of candidates identified in Table 2.

Identification of AroT. In Table 2, the protein subsequently identified as aromatic AT, AroT, attracted attention since among the substrates assayed it had the highest activities with O-Leu and L-Glu as substrate. McHardy et al. (19), who termed this gene *pat*, observed an auxotrophy for Leu/Ile/Phe (supplied together) when inactivated in an *ilvE* background, suggesting that the gene under consideration encodes an aromatic AT. We therefore assayed for activity with the substrates phenylpyruvate (O-Phe) and 4-hydroxyphenylpyruvate (O-Tyr) using L-Glu as an amino donor. The specific activities ($\mu\text{mol min}^{-1}$ mg of protein⁻¹) were 13.6 (O-Phe) and 8.8 (O-Tyr), respectively, confirming the function of the protein as an aromatic AT. The detectable activity with O-Leu as substrate (Table 2) is not surprising since the aromatic and branched-chain amino acids share a strong hydrophobicity. Also, aromatic amino acid AT TyrB of *E. coli* was shown to exhibit weak activity with O-Leu (25). Based on the finding that the branched-chain AT IlvE of *E. coli* shows activities for the formation of O-Phe and O-Tyr (7) and the mutant study with *C. glutamicum* (19), we also assayed IlvE of *C. glutamicum* for its specificity toward aromatic substrates. A remarkably high activity ($\mu\text{mol min}^{-1}$ mg of protein⁻¹) of 10.7 was obtained with O-Phe as substrate and 2.4 with O-Tyr. This might explain that the single *pat* inactivation did not result in an aromatic amino acid requirement (19).

Identification of AspT. In further assays with the other isolated AT proteins we searched for the aspartate AT, which is of prime interest for the synthesis of the aspartate-derived amino acids (14). These enzymes belong to the class I AT proteins (20), of which *C. glutamicum* possesses nine candidates (19). Since the function for three of them has been identified (9; this study), we assayed selected ATs with L-Asp and O-Glu as the substrate. With the protein encoded by NCgl0237 an activity was found. It was $10.7 \mu\text{mol min}^{-1}$ mg of protein⁻¹ identifying the protein as AspT.

Identification of AlaT. Another AT of interest is that converting pyruvate to L-alanine. The reason is that L-alanine is occasionally formed as by-product during L-lysine or L-valine production with *C. glutamicum* and knowledge of alanine ATs in general is scanty. For instance, the corresponding gene of *E. coli* has not yet been identified. In Table 2 we observed one

AT encoded by NCgl2747 which uses O-But as substrate together with any of the four amino donors assayed. We therefore deleted the gene in the WT to generate WT ΔalaT . Growth of this mutant was retarded on minimal medium CGXII (Fig. 3). When all 20 amino acids were supplied together, this complemented the growth defect. Further assays identified that L-Ala alone fully restores growth (Fig. 3). Interestingly, the auxotrophy was only apparent on agar plates (see Discussion). The enzyme assay subsequently performed confirmed L-alanine formation as the major activity. With pyruvate as substrate and L-Glu as amino donor the specific activity ($\mu\text{mol min}^{-1}$ mg of protein⁻¹) was 26.6, instead of 5.4 with O-But as substrate (Table 2). Furthermore, with pyruvate as substrate it was 1.8 with L-Asp as amino donor, 0.2 with L-Gln as amino donor, and 8.4 with aminobutyrate as amino donor. This rather broad amino donor specificity clearly distinguishes AlaT from all other ATs of *C. glutamicum* (see also Table 2).

Identification of cysteine desulfurases. Three of the AT proteins (NCgl1500, NCgl1184, and NCgl1022) were isolated from *E. coli* as yellowish proteins. Their absorption spectrum identified them as containing pyridoxal-5'-phosphate (not shown). These proteins belong to class V of ATs containing phosphoserine ATs and cysteine desulfurases. As already concluded from the genomic context (19), the proteins might be involved in the synthesis of Fe-S complexes. In an enzyme assay we observed high alanine generation from L-cysteine accompanied by the unpleasant smell of sulfur-derived compounds with the proteins encoded by NCgl1500 and NCgl1022 identifying them as cysteine desulfurases. The determined specific activities of 0.35 and 0.04 ($\mu\text{mol min}^{-1}$ mg of protein⁻¹), respectively, were comparable to the cysteine desulfurase IscS of *E. coli* (23).

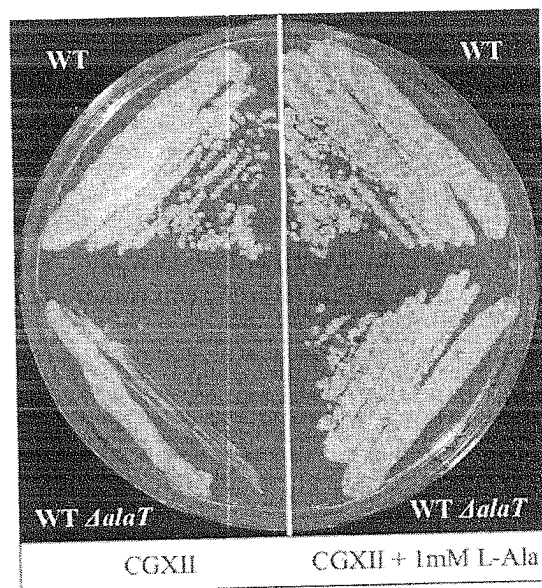


FIG. 3. L-Ala synthesis by AlaT. Only on the right side did the salt medium CGXII contain L-Ala. At the top is shown the WT, and at the bottom the WT with *alaT* deleted is shown.

TABLE 4. Overview of the ATs of *C. glutamicum*

NCgl	Gene	Alias(es)	Class ^a		Enzyme ^b	Cellular function ^b
			Mehta et al.	Batemann et al.		
NCgl0215	<i>aroT</i>	<i>pat, tyrB</i>	I	I, II	Aromatic amino acid AT*	Aromatic amino acid synthesis (19)
NCgl0237	<i>aspT</i>		I		Aspartate AT*	Aspartate synthesis
NCgl0422	<i>hemL</i>		II	III	Glutamate semialdehyde AT	Uroporphyrinogen synthesis
NCgl0462			II	III	Butanoate metabolism	4-Aminobutyrate aminotransferase
NCgl0753	<i>pdxR</i>		I		Pyridoxamine-P AT	Pyridoxal-P synthesis* (19)
NCgl0780			I	I, II		
NCgl0794	<i>serC</i>				Phosphoserine AT	Serine synthesis* (24)
NCgl1022			IV	V	Cysteine desulfurase*	Involved in NAD synthesis
NCgl1058	<i>dapC</i>		I	I, II	Succinyl-diaminopimelate AT* (9)	Lysine synthesis* (9)
NCgl1184			IV	V	Cysteine desulfurase	Assembly of FeS complex of electron transfer flavoprotein
NCgl1343	<i>argD</i>		II	I	Acetylornithine AT*	Arginine synthesis* (29)
NCgl1500	<i>sufS</i>		IV	V	Cysteine desulfurase*	Assembly of FeS complexes
NCgl2020	<i>hisC</i>		I	I, II	Histidinol phosphate AT	Histidine synthesis* (19)
NCgl2123	<i>ilvE</i>		III	IV	Branched-chain amino acid AT*	BCAA synthesis* (26)
NCgl2227	<i>metC</i>	<i>aecD</i>	I	I, II	Cystathionine β -lyase* (15)	Methionine synthesis
NCgl2355			II	III		
NCgl2491			III	IV		Glycine cleavage?
NCgl2510	<i>avtA</i>		I	I, II	Valine-pyruvate AT*	
NCgl2515	<i>bioA</i>		II	III	AdoMet-aminooxononanoate AT	Biotin synthesis* (10)
NCgl2747	<i>alaT</i>		I	IV	Alanine AT*	Alanine synthesis*

^a The assignments in the left column are according to Mehta et al. (20); those in the right column are according to Batemann et al. (1).

^b An asterisk indicates experimentally derived data on enzyme activity or function, and a reference is given in parentheses for data obtained from previous studies.

Activities of ArgD, DapC, and HemL. The AT DapC, responsible for L-lysine synthesis, has been identified by activity determinations in crude extracts (9), and the AT ArgD, responsible for L-arginine synthesis, is known due to its clustering with *arg* genes in *C. glutamicum* (29). These enzymes aminate the structurally related substrates succinyl-diaminopimelate and acetyl-ornithine, and in *E. coli* both enzymes have activities with the two substrates which has led to confusion with respect to the assignment of the proteins (3, 16). Interestingly, inactivation of *dapC*, together with *argD*, in *C. glutamicum* still enables growth of the mutant without supplementation (9), requiring an even further AT of sufficient activity to sustain lysine-independent growth. We therefore followed the proposal of A. Tauch (University of Bielefeld, Germany) that HemL might also use succinyl-diaminopimelate as substrate and compared the activities of the proteins in question. The activities (in $\mu\text{mol min}^{-1} \text{mg of protein}^{-1}$) with succinyl-diaminopimelate were 1.2 with DapC, 0.006 with ArgD, and <0.001 with HemL. With acetyl-ornithine they were <0.001 with DapC, 6.4 with ArgD, and 0.046 with HemL. It is doubtful whether the weak HemL activity contributes to L-lysine synthesis.

DISCUSSION

Table 4 provides a complete overview of the ATs plus some PLP-containing proteins as results from the various approaches based on genome information for *C. glutamicum* and functional studies. The PLP-containing MetC (AecD) is not an AT, but it has β -lyase activity toward cystathionine (27) or the unnatural amino acid *S*-(2-aminoethyl)-D,L-cysteine (35). We also did not find any AT activity with NCgl2491, which is adjacent in the genome to a putative T-protein of a glycine cleavage system. Three of the proteins were isolated as colored proteins and contain firmly bound PLP. They are likely to carry

out a β -elimination, which we have demonstrated for the proteins encoded by NCgl1500 and NCgl1022. These are desulfurases that cleave L-cysteine to form alanine together with an enzyme cysteinyl persulfide intermediate (21). The mobilized sulfur is used for a number of processes, such as Fe-S cluster assembly, as well as the synthesis of thiamine, lipoic acid, or thionucleosides in tRNA. Since NCgl1500 is part of the well-conserved *sufABCD*S operon of bacteria and plants, assisting in the sulfur transfer pathway for Fe-S cluster assembly (23), we denote this protein SufS. SufS is an abundant protein in *C. glutamicum* (30), which substantiates the idea that SufS represents the major activity for Fe-S cluster generation. The gene NCgl1184 is arranged in synteny within the *Corynebacteriaceae* with genes of lipid synthesis and electron-transferring flavoproteins, which might eventually transfer the reducing equivalents formed during the oxidation of fatty acyl-coenzyme A (CoA) to *trans*- Δ^2 -enoyl-CoA to the membrane-bound quinone pool, and the quinone oxidoreductase catalyzing this latter activity is also an Fe-S cluster protein. Thus, it appears that a more specific sulfur-providing pathway is necessary for synthesis of the Fe-S cluster in the quinone oxidoreductase. The same holds true for the third desulfurase (NCgl1022), which is clustered together with quinolinate synthetase A, an Fe-S protein required for NAD synthesis.

Two of the newly identified ATs are clearly separate from the others: AvtA and AlaT. Bioinformatic analyses currently recognize the AvtA structure as similar to the class I (20) or class I/II structures of ATs (1), which represent the most common types of ATs present in *C. glutamicum*. Nevertheless, AvtA is the only AT with exceptionally high activity toward L-alanine as an amino donor instead of preferably using L-glutamate. As 2-oxo-acid, it preferably accepts O-But with highest activity and affinity, followed by O-Val (Table 2). The

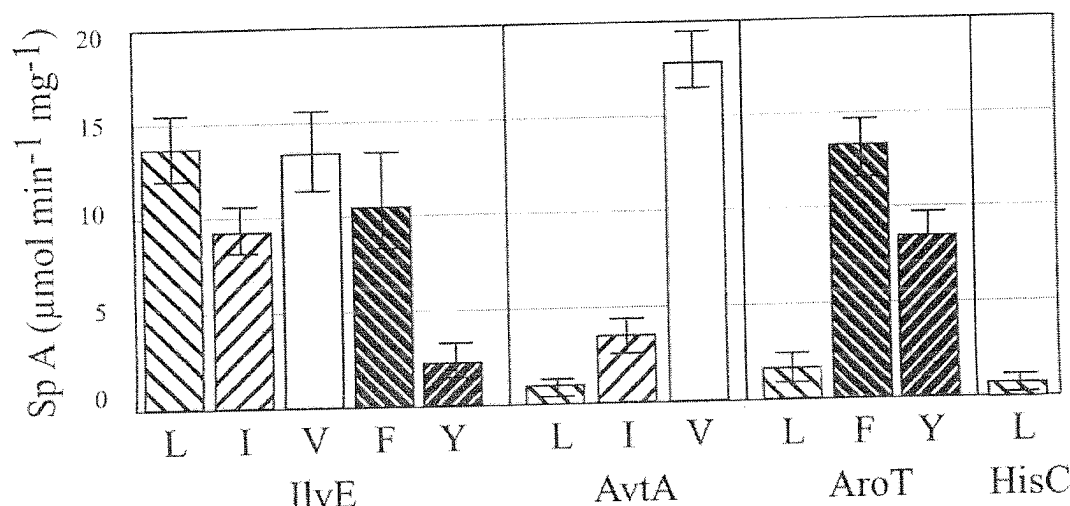


FIG. 4. Comparison of the activities of the ATs showing overlapping substrate specificities for the in vitro formation of the branched-chain and aromatic amino acids. 2020 is the NCgl number of the protein (see Table 4). The amino donor for IleV, AroT, and 2020 was L-glutamate. The amino donor for AvaT was L-alanine.

enzyme corresponds to the transaminase C activity already found in extracts of *C. glutamicum* (17). A similar enzyme activity is present in *E. coli*, which has been demonstrated to use L-alanine or aminobutyrate as equivalent amino donors to aminate O-Val (28). However, the in vivo function of AvaT is difficult to assign, since in *E. coli* and *C. glutamicum* as well, the *avaT* mutation has no phenotype. It could be that AvaT is involved in the adjustment of amino acid pool sizes in *C. glutamicum* rather than fulfilling a specific biosynthesis function.

The other AT functionally distinguished and identified in the genome of *C. glutamicum* is AlaT. Recent AT classifications classify AlaT into family IV, of which a total of only three proteins are present in *C. glutamicum* (Table 4). AlaT is characterized by a broad specificity for the amino donor, in the order Glu > But > Asp (with pyruvate as acceptor) and Glu > Ala > Asp (with O-But as acceptor). Knowledge of bacterial Ala ATs is limited, and one other AlaT is known from *Pyrococcus furiosus*, where it serves as an electron sink to produce L-Ala during fermentation of sugars (36). Of the *C. glutamicum* ATs, AlaT has the highest identity (37%) to the AlaT of *P. furiosus*. Using these structures as seed information, we propose that *yfbQ* of *E. coli* is an alanine transaminase. Interestingly, the *alaT* phenotype of *C. glutamicum*, which is an L-alanine requirement on plates (Fig. 3), is not present during growth of the same clone in liquid culture CGXII (not shown). This could be due to the overlapping AT activities and at the same time a different AT regulation under the two growth conditions used.

As already mentioned in the introduction, there is a strong overlap of transamination activity for the hydrophobic substrates of *E. coli*, which is also present in *C. glutamicum* (Fig. 4). An early article already reported on two separate activities in *C. glutamicum* for the transamination of O-Phe and O-Tyr (6), and these two activities were not considered likely to be identical to IleV (32). This agrees with the recent observation that an *ihvE*, *aroT* double mutant (19) does not require L-Tyr for

growth. Therefore, in addition to AroT and IleV, which both have comparable activities for O-Phe (Fig. 4), a further still unknown activity for L-Tyr is required. In *E. coli* AspC and TyrB (together originally named transaminase A) are similar in many respects, and both have activity toward aromatic amino or oxo acids (11). We assayed AspC of *C. glutamicum* with aromatic amino acids and AroT with oxo acids, but in neither case was any activity detected (not shown). Therefore, the third AT active in *C. glutamicum* with O-Tyr still has to be identified. Also, for the branched-chain amino acids there is overlapping AT activity. However, this depends very much on the specific amino acid in question. For instance, in vivo, L-Ile appears to be exclusively synthesized via IleV (Fig. 1), and the activity due to AvaT (Fig. 4) might be too low to sustain growth of the *ihvE* mutant. The situation with L-Val is different, since the high AvaT activity with this substrate (Fig. 4) is apparently sufficient to enable significant growth, and only upon deletion of both AvaT and IleV is there an absolute requirement for L-Val (Fig. 2). Although there are three additional proteins—AvaT, AroT, and HisC—acting on 2-oxo-isocaproate to synthesize L-Leu (Fig. 4), these activities are apparently too weak to sustain significant growth. Whereas it is largely now clear which of the overlapping activities contributes to aromatic and branched-chain amino acid synthesis, this is less clear for aminobutyrate formation. The largest activities have IleV and AvaT (Table 3). However, as is evident from the present study, there are a number of additional activities present in *C. glutamicum*. This is not unexpected, considering the broad substrate specificity and versatility of transaminating activities.

ACKNOWLEDGMENTS

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REFERENCES

- Bateman, A., L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E. L. Sonnhammer, D. J. Studholme, C. Yeats, and S. R. Eddy. 2004. The Pfam protein families database. *Nucleic Acids Res.* 32:D138–D141. [Online.] <http://www.sanger.ac.uk/Software/Pfam/Version17.0>.
- Berg, C. M., W. A. Whalen, and L. B. Archambault. 1983. Role of alanine-valine transaminase in *Salmonella typhimurium* and analysis of an *avtA::Tn5* mutant. *J. Bacteriol.* 155:1009–1014.
- Cox, R. J., and P. S. Wang. 2001. Is N-acetylornithine aminotransferase the real N-succinyl-L-lysine aminotransferase in *E. coli* and *M. smegmatis*? *J. Chem. Soc. Perkin Trans.* 1:2006–2008.
- Eggeling, L., S. Morbach, and H. Sahm. 1997. The fruits of molecular physiology: engineering the L-isoleucine biosynthesis pathway in *Corynebacterium glutamicum*. *J. Biotechnol.* 56:167–182.
- Eggeling, L., and M. Bott. 2005. *Handbook of Corynebacterium glutamicum*. CRC Press, Inc., Boca Raton, Fla.
- Fazel, A. M., and R. A. Jensen. 1979. Aromatic aminotransferases in coryneform bacteria. *J. Bacteriol.* 140:580–587.
- Gelfand, D. H., and R. A. Steinberg. 1977. *Escherichia coli* mutants deficient in the aspartate and aromatic amino acid aminotransferases. *J. Bacteriol.* 130:429–440.
- Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc. Natl. Acad. Sci. USA* 87:4645–4649.
- Hartmann, M., A. Tauch, L. Eggeling, B. Bathe, B. Mückel, A. Pühler, and J. Kalinowski. 2003. Identification and characterization of the last two unknown genes, *dapC* and *dapF*, in the succinylase branch of the L-lysine biosynthesis of *Corynebacterium glutamicum*. *J. Biotechnol.* 104:199–211.
- Hatakeyama, K., K. Hohama, A. A. Vertes, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1993. Genomic organization of the biotin biosynthetic genes of coryneform bacteria: cloning and sequencing of the *bioA-bioI* genes from *Brevibacterium flavum*. *DNA Seq.* 4:177–184.
- Hayashi, H., K. Inoue, T. Nagata, S. Kuramitsu, and H. Kagamiyama. 1993. *Escherichia coli* aromatic amino acid aminotransferase: characterization and comparison with aspartate aminotransferase. *Biochemistry* 32:12229–12239.
- Ilag, L. L., D. Jahn, G. Eggertsson, and D. Soll. 1991. The *Escherichia coli hemI* gene encodes glutamate 1-semialdehyde aminotransferase. *J. Bacteriol.* 173:3408–3413.
- John, R. A. 1995. Pyridoxal phosphate-dependent enzymes. *Biochim. Biophys. Acta* 1248:81–96.
- Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkowski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Kramer, B. Linke, A. C. McHardy, F. Meyer, B. Mückel, W. Pfeifferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegand, and A. Tauch. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* 104:5–25.
- Kim, J. W., H. J. Kim, Y. Kim, M. S. Lee, and H. S. Lee. 2001. Properties of the *Corynebacterium glutamicum metC* gene encoding cystathionine beta-lyase. *Mol. Cells* 11:220–225.
- Ledwidge, R., and J. S. Blanchard. 1999. The dual biosynthetic capability of N-acetylornithine aminotransferase in arginine and lysine biosynthesis. *Biochemistry* 38:3019–3024.
- Leyval, D., D. Uy, S. Delaunay, J. L. Goergen, and J. M. Engasser. 2003. Characterisation of the enzyme activities involved in the valine biosynthetic pathway in a valine-producing strain of *Corynebacterium glutamicum*. *J. Biotechnol.* 104:241–252.
- Link, A. J., D. Phillips, and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol.* 179:6228–6237.
- McHardy, A., A. Tauch, C. Rückert, A. Pühler, and J. Kalinowski. 2003. Genome-based analysis of biosynthetic aminotransferase genes of *Corynebacterium glutamicum*. *J. Biotechnol.* 104:229–240.
- Mehla, P. K., T. I. Hale, and P. Christen. 1993. Aminotransferases: demonstration of homology and division into evolutionary subgroups. *Eur. J. Biochem.* 214:549–561.
- Mihara, H., and N. Esaki. 2002. Bacterial cysteine desulfurases: their function and mechanisms. *Appl. Microbiol. Biotechnol.* 60:12–23.
- Morbach, S., Sahm, H., and L. Eggeling. 1996. L-isoleucine production with *Corynebacterium glutamicum*: further flux increase and limitation of export. *Appl. Environ. Microbiol.* 62:4345–4351.
- Outen, F. W., M. J. Wood, F. M. Munoz, and G. Storz. 2003. The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. *J. Biol. Chem.* 278:45713–45719.
- Peters-Wendisch, P., M. Stolz, H. Ertterich, N. Kennerknecht, H. Sahm, and L. Eggeling. Metabolic engineering of *Corynebacterium glutamicum* for L-serine production. *Appl. Environ. Microbiol.*, in press.
- Powell, J. T., and J. F. Morrison. 1978. Role of the *Escherichia coli* aromatic amino acid aminotransferase in leucine biosynthesis. *J. Bacteriol.* 136:1–4.
- Radmacher, E., A. Vaisikova, U. Burger, K. Krumbach, H. Sahm, and L. Eggeling. 2002. Linking central metabolism with increased pathway flux: L-valine accumulation by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 68:2246–2250.
- Rossol, L., and A. Pühler. 1992. The *Corynebacterium glutamicum uedC* gene encodes a C-S lyase with α - β -elimination activity that degrades aminoethylcysteine. *J. Bacteriol.* 174:2963–2977.
- Rudman, D., and A. Meister. 1953. Transamination in *Escherichia coli*. *J. Biol. Chem.* 200:591–604.
- Sakanyan, V., P. Petrosyan, M. Lecocq, A. Boyen, C. Legrain, M. J. Demarez, N. Hallet, and N. Glansdorff. 1996. Genes and enzymes of the acetyl cycle of arginine biosynthesis in *Corynebacterium glutamicum*: enzyme evolution in the early steps of the arginine pathway. *Microbiology* 142:99–108.
- Schaffer, S., B. Weil, V. D. Nguyen, G. Dongmann, K. Gunther, M. Nikolaus, T. Hermann, and M. Bott. 2001. A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum*. *Electrophoresis* 22:4404–4422.
- Schäfer, A., A. Tauch, W. Jaeger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145:69–73.
- Shio, I., and S. I. Sugimoto. 1981. Regulation at metabolic branch points of aromatic amino acid biosynthesis in *Brevibacterium flavum*. *Agric. Biol. Chem.* 45:2197–2207.
- Skerra, A. 1994. Use of the production of a murine antibody fragment in *Escherichia coli*. *Gene* 151:131–135.
- Tauch, A., O. Kirchner, B. Löffler, S. Götker, A. Pühler, and J. Kalinowski. 2002. Efficient electrotransformation of *Corynebacterium diphtheriae* with a mini-replicon derived from the *Corynebacterium glutamicum* plasmid pGAI. *Curr. Microbiol.* 45:362–367.
- Thierbach, G., J. Kalinowski, B. Bachmann, and A. Pühler. 1990. Cloning of a DNA fragment from *Corynebacterium glutamicum* conferring aminoethylcysteine resistance and feedback resistance to aspartokinase. *Appl. Microbiol. Biotechnol.* 32:443–448.
- Ward, D. E., S. W. Kengen, J. van Der Oost, and W. M. de Vos. 2000. Purification and characterization of the alanine aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its role in alanine production. *J. Bacteriol.* 182:2559–2566.
- Whalen, W. A., and C. M. Berg. 1982. Analysis of an *avtA::Mu d1(Ap lac)* mutant: metabolic role of transaminase C. *J. Bacteriol.* 150:739–746.
- Wilhelm, C., L. Eggeling, A. Nassenstein, C. Jeben, I. Eggeling, and H. Sahm. 1989. Limitations during hydroxybutyrate conversion to isoleucine with *Corynebacterium glutamicum* as analysed by the formation of by-products. *Appl. Microbiol. Biotechnol.* 172:458–462.

EXHIBIT B



US006040160A

United States Patent [19]
Kojima et al.

[11] **Patent Number:** **6,040,160**
 [45] **Date of Patent:** **Mar. 21, 2000**

[54] **METHOD OF PRODUCING L-LYSINE BY
 FERMENTATION**

[75] Inventors: **Hiroyuki Kojima; Yuri Ogawa; Kazue
 Kawamura; Konosuke Sano**, all of
 Kawasaki, Japan

[73] Assignee: **Ajinomoto Co., Inc.**, Tokyo, Japan

[21] Appl. No.: **08/648,010**

[22] PCT Filed: **Nov. 28, 1994**

[86] PCT No.: **PCT/JP94/01994**

§ 371 Date: **May 29, 1996**

§ 102(e) Date: **May 29, 1996**

[87] PCT Pub. No.: **WO95/16042**

PCT Pub. Date: **Jun. 15, 1995**

[30] **Foreign Application Priority Data**

Dec. 8, 1993 [JP] Japan 5-308397

[51] **Int. Cl.⁷** **C12P 13/08; C12N 1/20;
 C12N 15/00; C07H 21/04**

[52] **U.S. Cl.** **435/115; 435/252.3; 435/254.11;
 435/320.1; 435/325; 536/23.2**

[58] **Field of Search** **435/115, 252.3,
 435/254.11, 320.1, 325; 536/23.2**

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,258,300 11/1993 Glassman et al. 435/240.4
 5,367,110 11/1994 Galili et al. 800/205
 5,545,545 8/1996 Gengenbach et al. 435/172.3

FOREIGN PATENT DOCUMENTS

0 435 132 7/1991 European Pat. Off. .
 19190 9/1993 WIPO 536/23.2
 WO 93/19190 9/1993 WIPO .
 11517 5/1994 WIPO 536/23.2
 WO 94/11517 5/1994 WIPO .

OTHER PUBLICATIONS

M. Hermann, et al., European Journal of Biochemistry, vol. 30, pp. 100-106, "Consequences of Lysine Oversynthesis in Pseudomonas Mutants Insensitive to Feedback Inhibition.

Lysine Excretion of Endogenous Induction of Lysine-Catabolic Pathway", 1972.

D.C. Bittel, et al., Am. Soc. Plant Physiologists, pp. 322-323, "Characterization of A Lysine-Intensive Form of Dihydrodipicolinate Synthase From Maize", 1992.

Valérie Frankard et al., "Two Feedback-Insensitive Enzymes of the Aspartate Pathway in *Nicotiana sylvestris*", Plant Physiol., vol. 99, pp. 1285-1293, 1992.

Orit Shaul et al., "Concerted Regulation of Lysine and Threonine Synthesis in Tobacco Plants Expressing Bacterial Feedback-Insensitive Aspartate Kinase and Dihydrodipicolinate Synthase", Plant Molecular Biology., vol. 23, pp. 759-768, 1993.

Michel Cassan et al, Nucleotide Sequence of IysC Gene Encoding the Lysine-sensitive Aspartokinase III of *Escherichia coli* K12.

Journal of Bacteriology, vol. 166, No. 1, pp. 297-300, Apr. 1986, F. Richaud, et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia Coli* dapA Gene".

Brock et al. (1973) Modification of amino acid composition of higher plants by mutation and selection. Caplus Access No: 1974:488102 Caplus, May 1973.

Primary Examiner—Bradley Sisson

Assistant Examiner—Einar Stole

Attorney, Agent, or Firm—Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

[57] **ABSTRACT**

A bacterium belonging to the genus *Escherichia*, which is transformed by introducing, into its cells, a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia* having mutation to desensitize feedback inhibition by L-lysine and a DNA coding for an aspartokinase III originating from a bacterium belonging to the genus *Escherichia* having mutation to desensitize feedback inhibition by L-lysine; preferably a bacterium belonging to the genus *Escherichia* in which a dihydrodipicolinate reductase gene and a diaminopimelate dehydrogenase gene originating from *Brevibacterium lactofermentum* (or a succinyl diaminopimelate transaminase gene and a succinyl diaminopimelate deacylase gene) are further enhanced, is cultivated in an appropriate medium, L-lysine is produced and accumulated in a culture thereof, and L-lysine is collected from the culture.

22 Claims, 18 Drawing Sheets

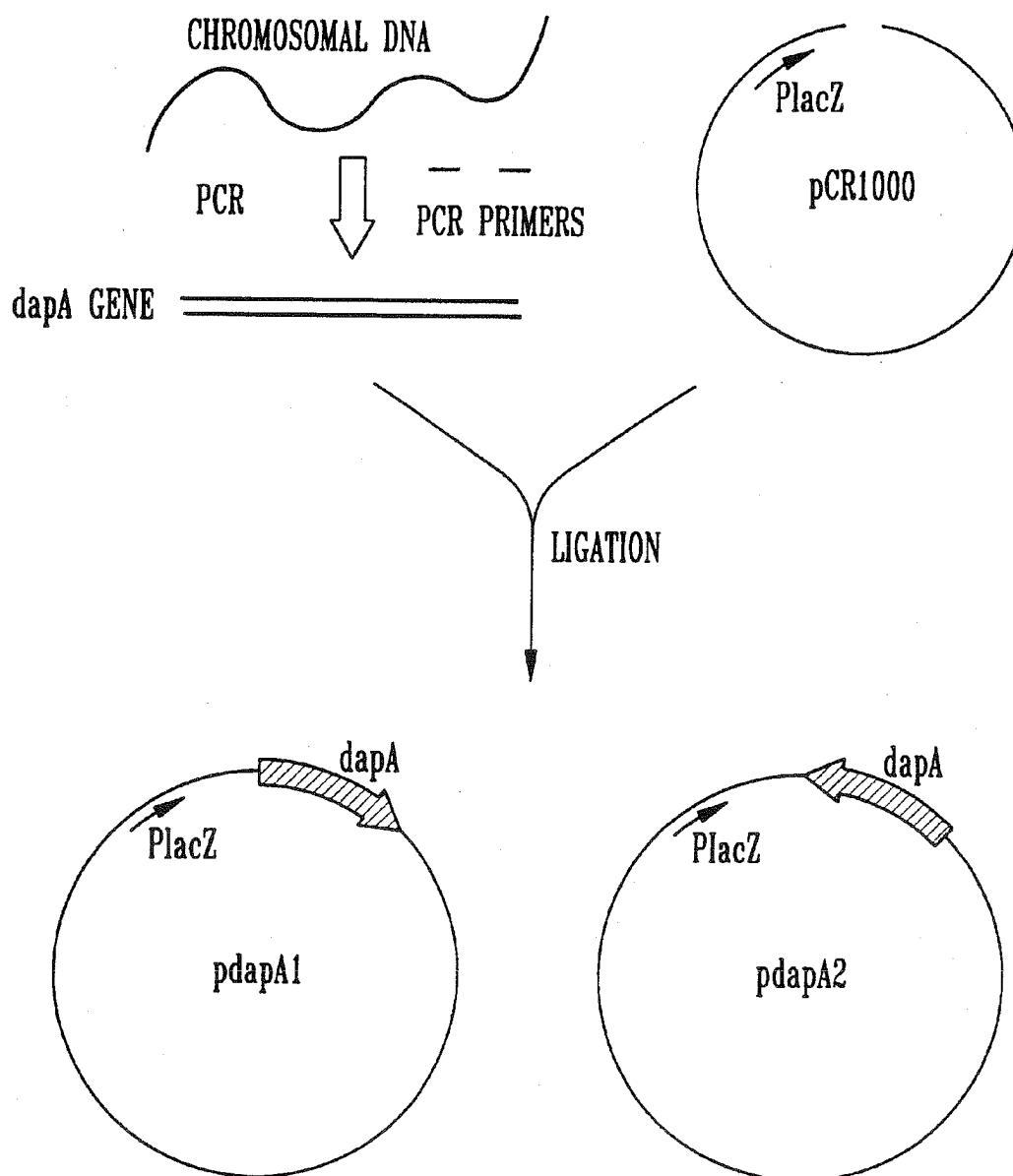
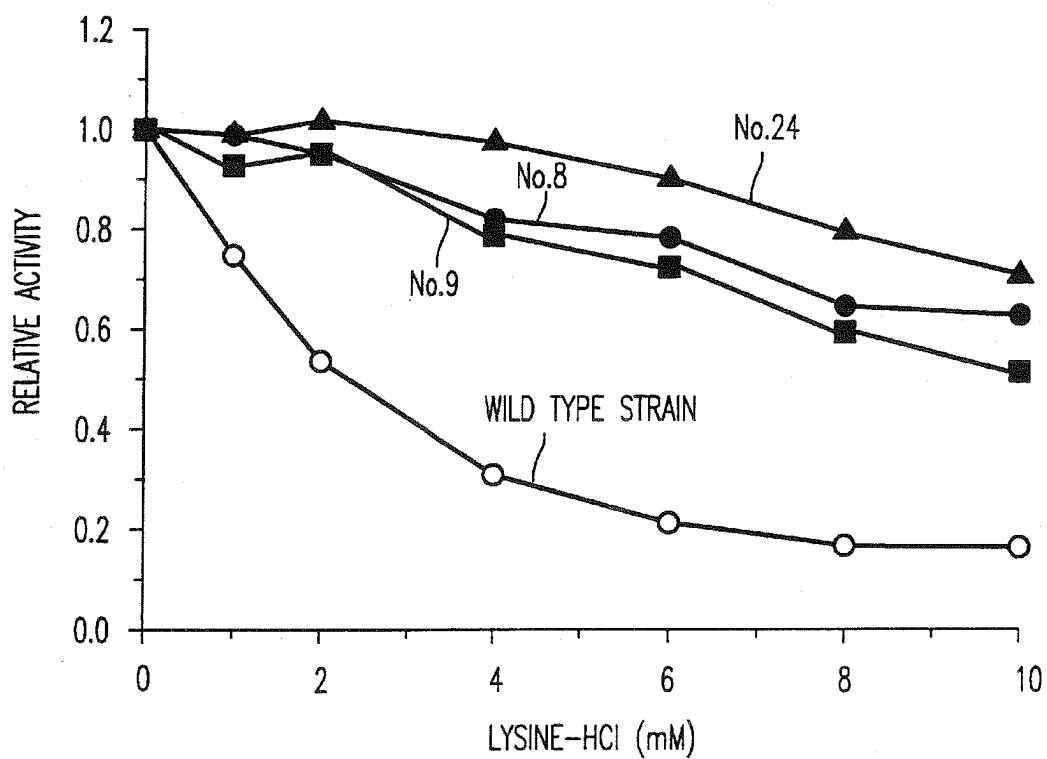
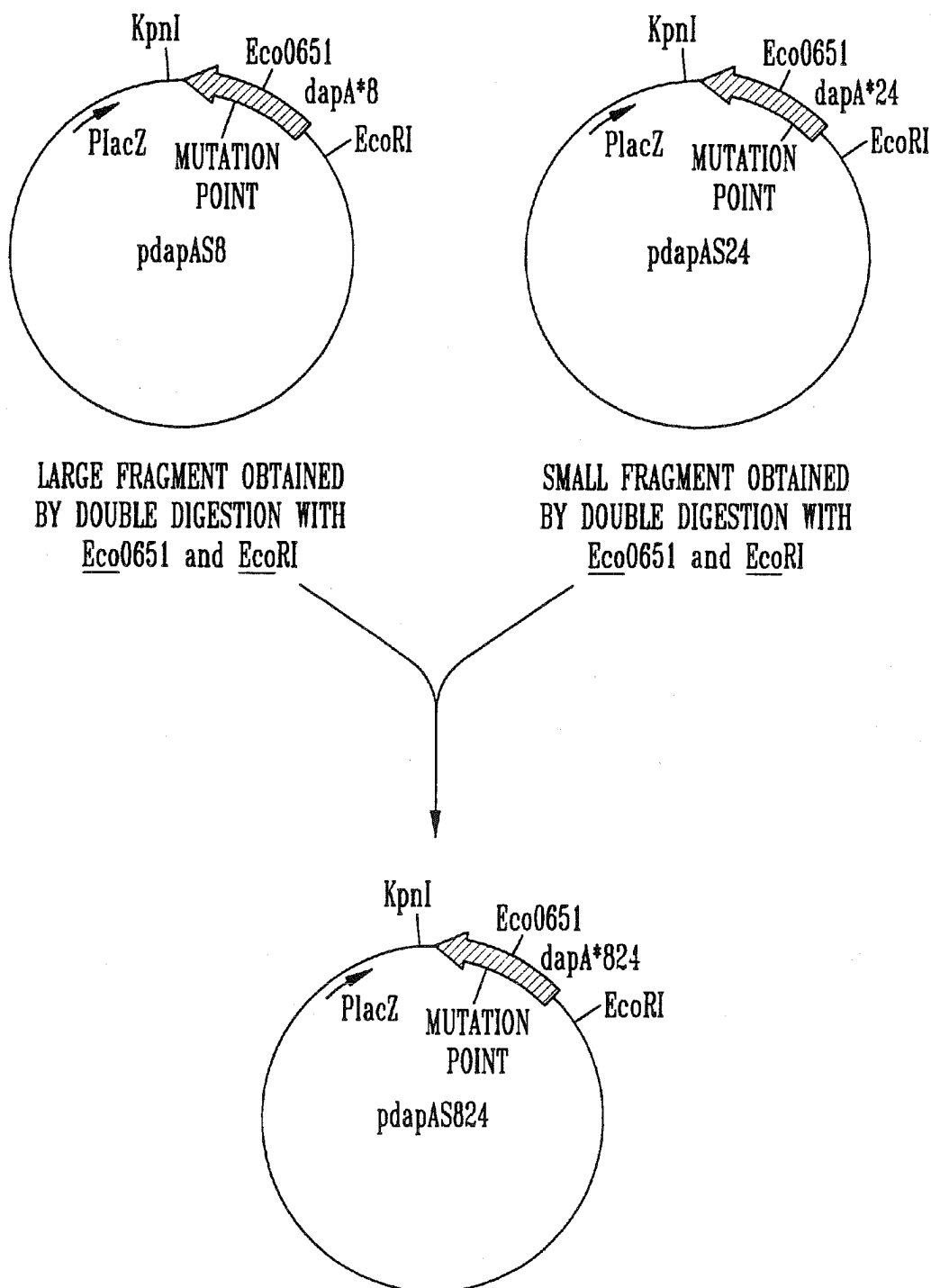
*FIG. 1*

FIG. 2

**FIG. 3**

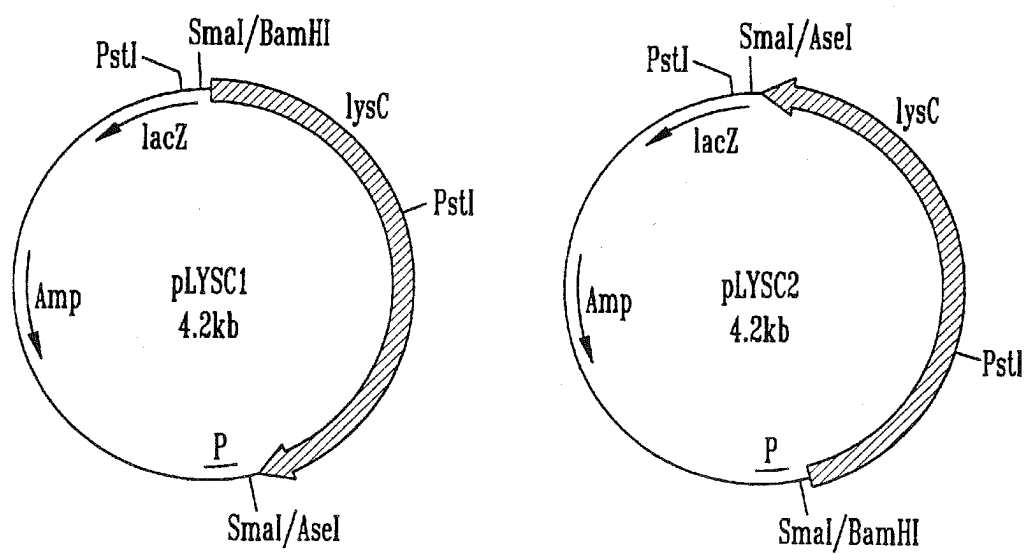
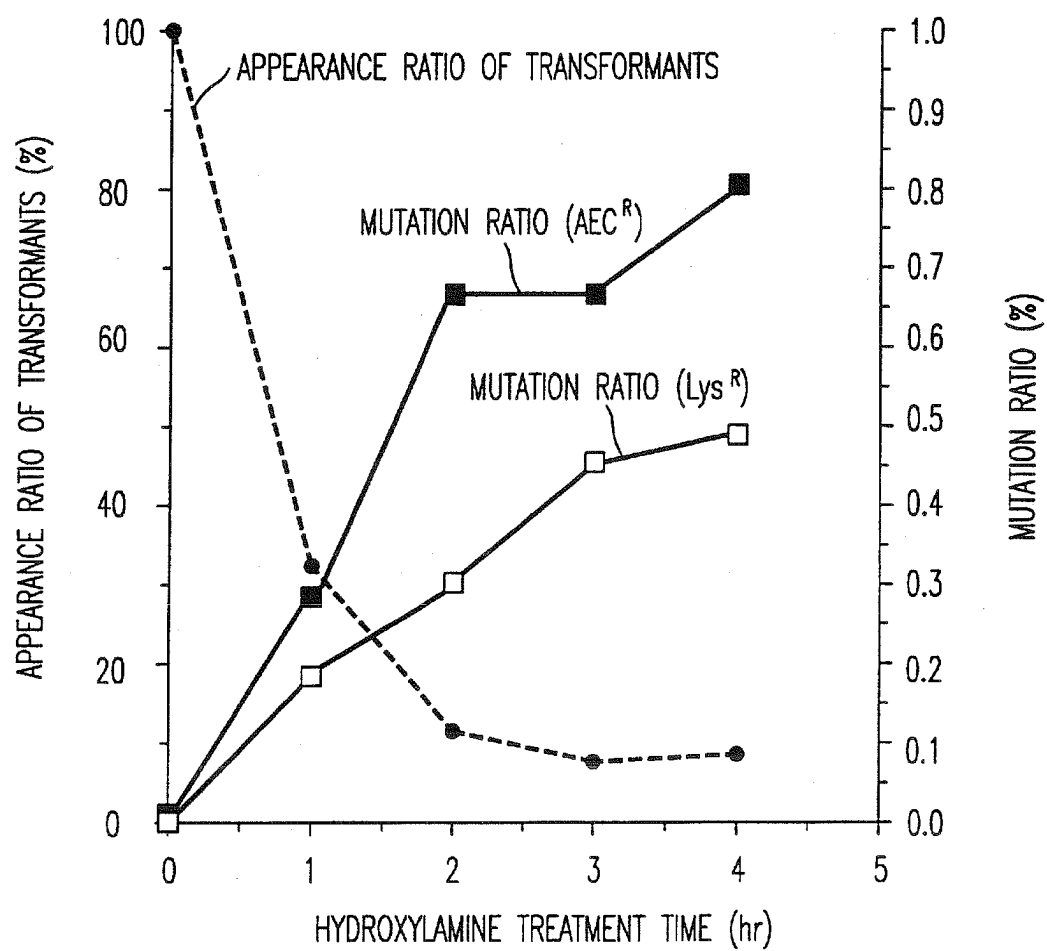
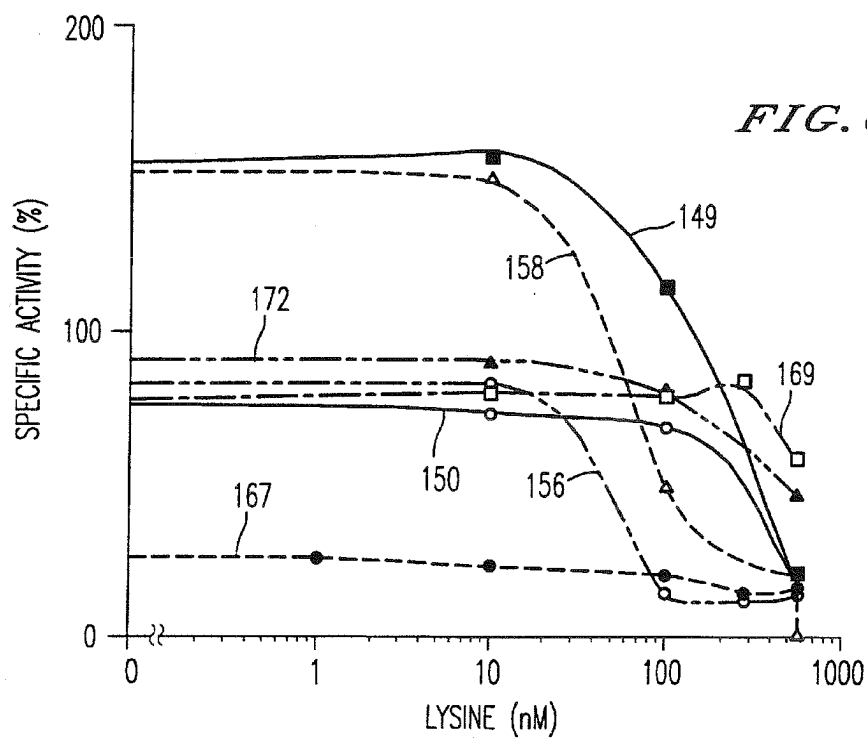
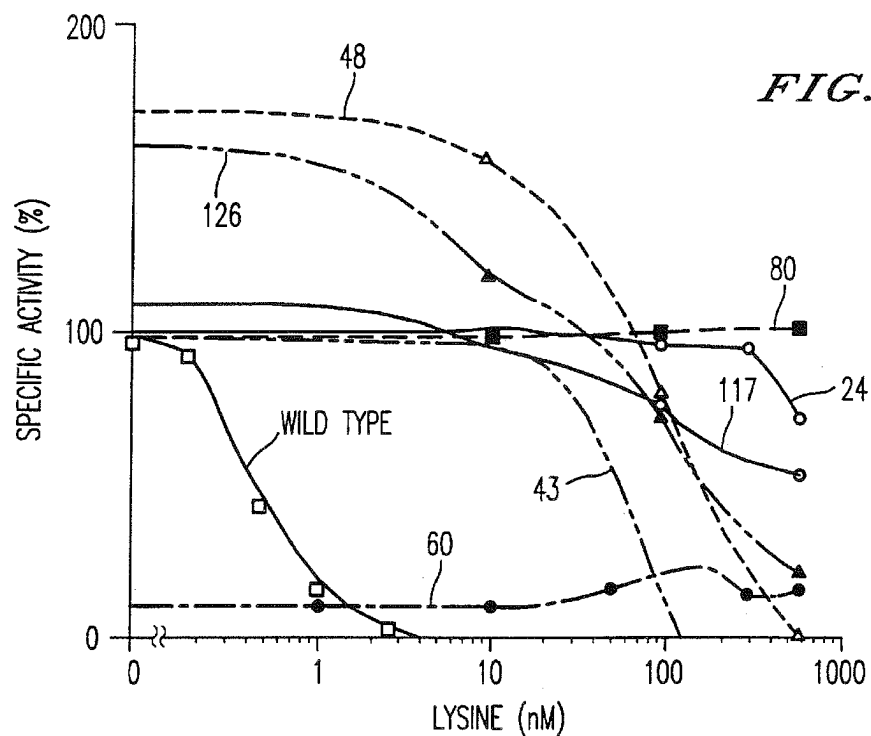
*FIG. 4*

FIG. 5

INTRODUCTION OF MUTATION WITH HYDROXYLAMINE





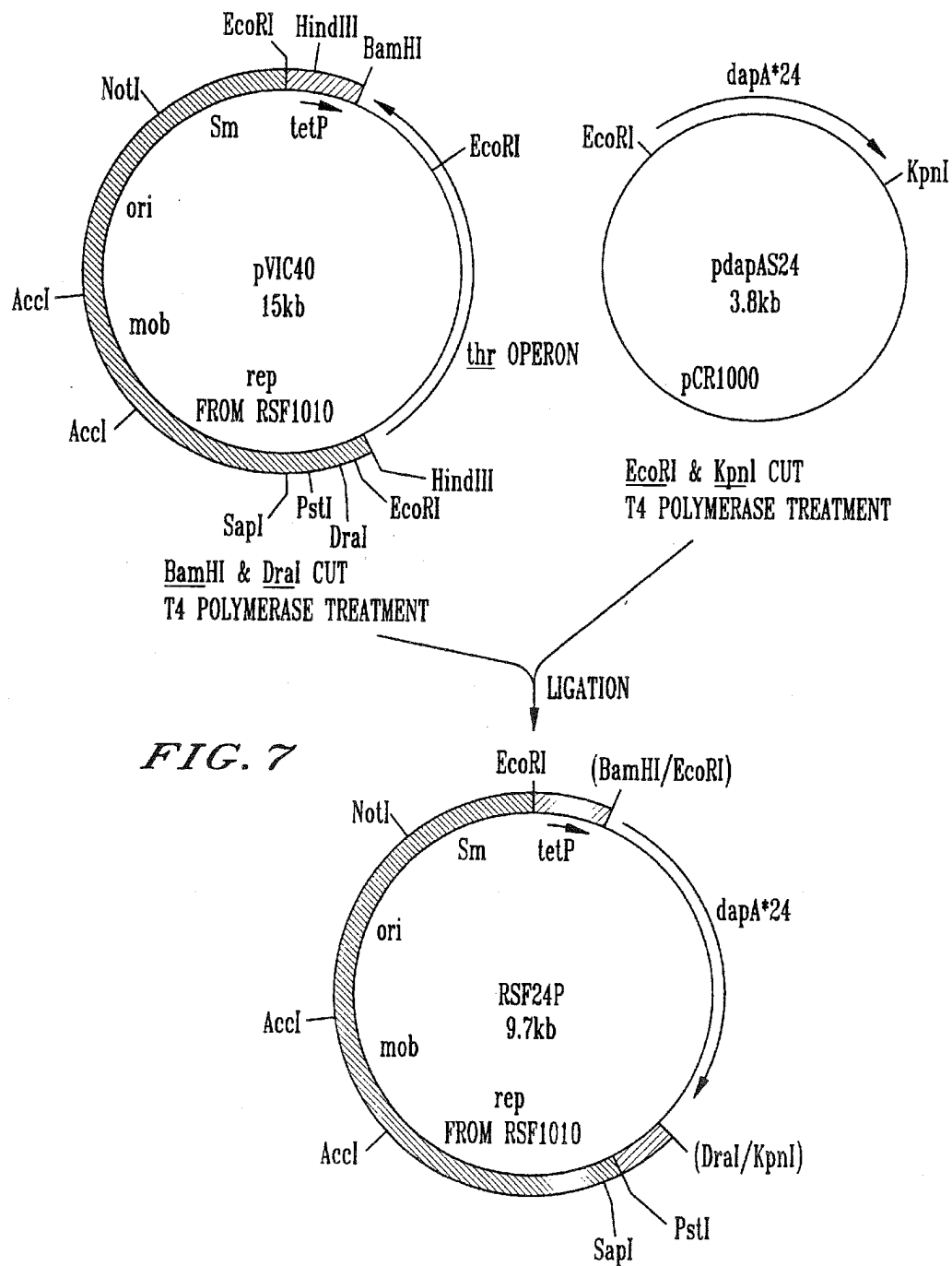
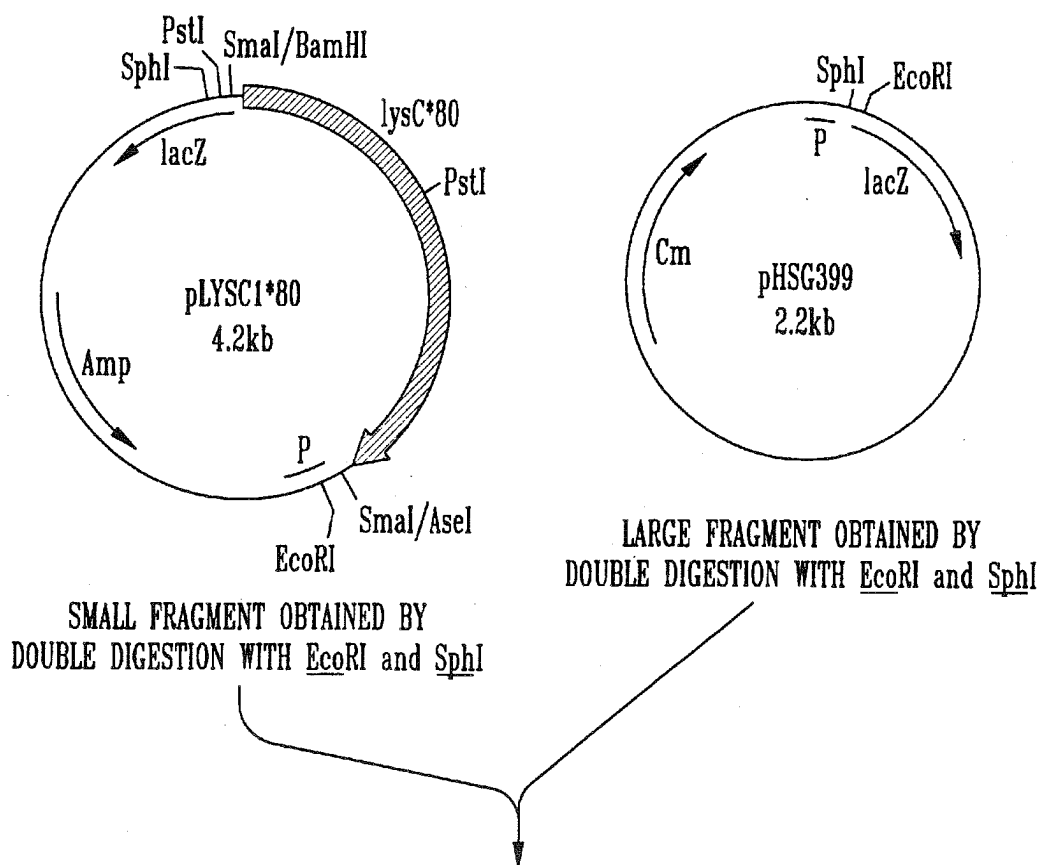
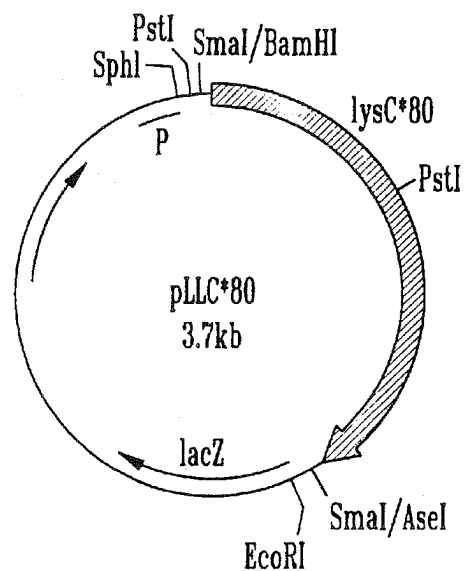
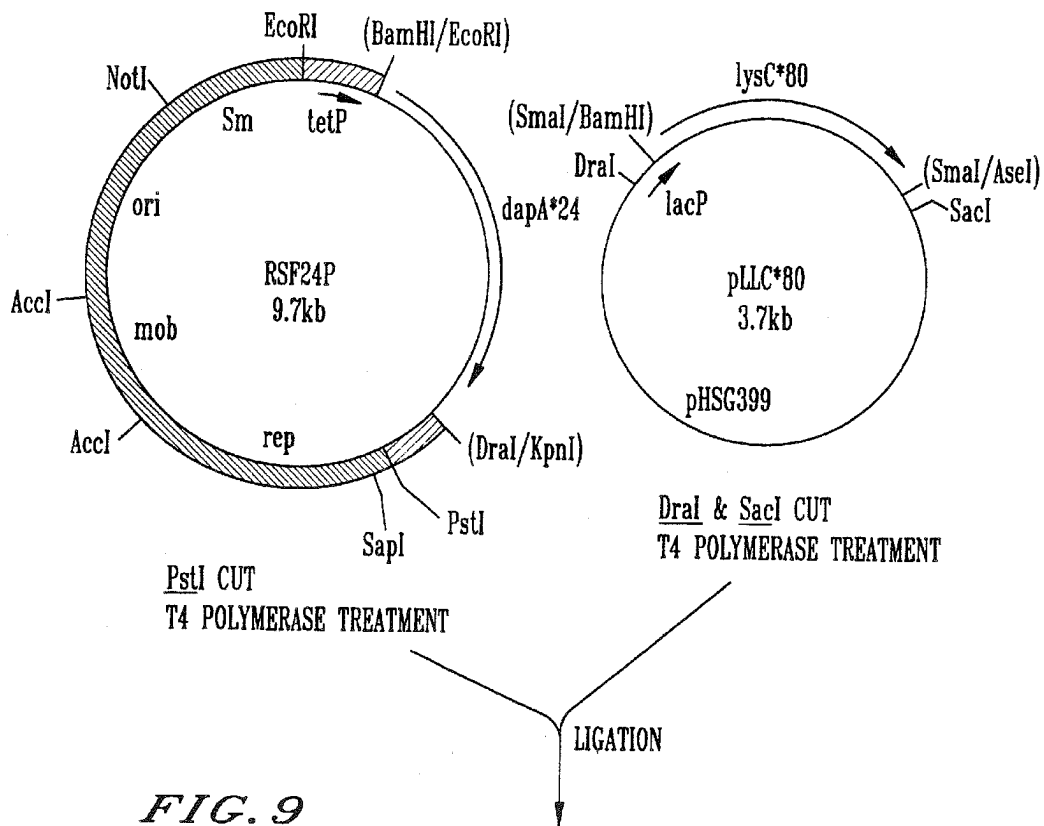
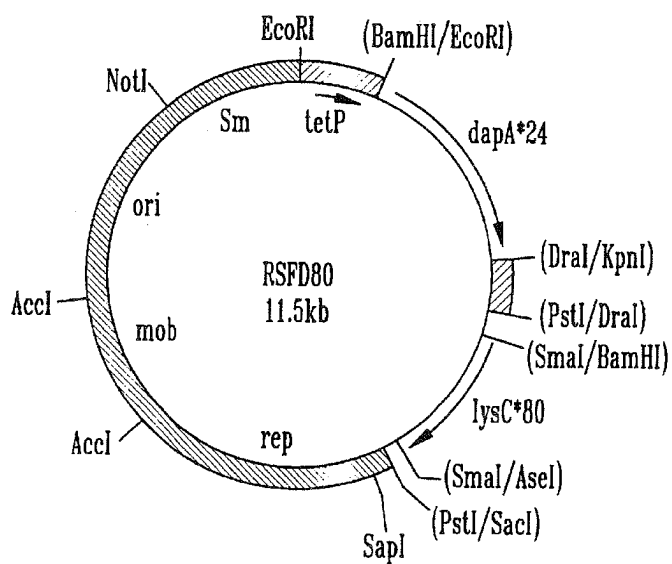
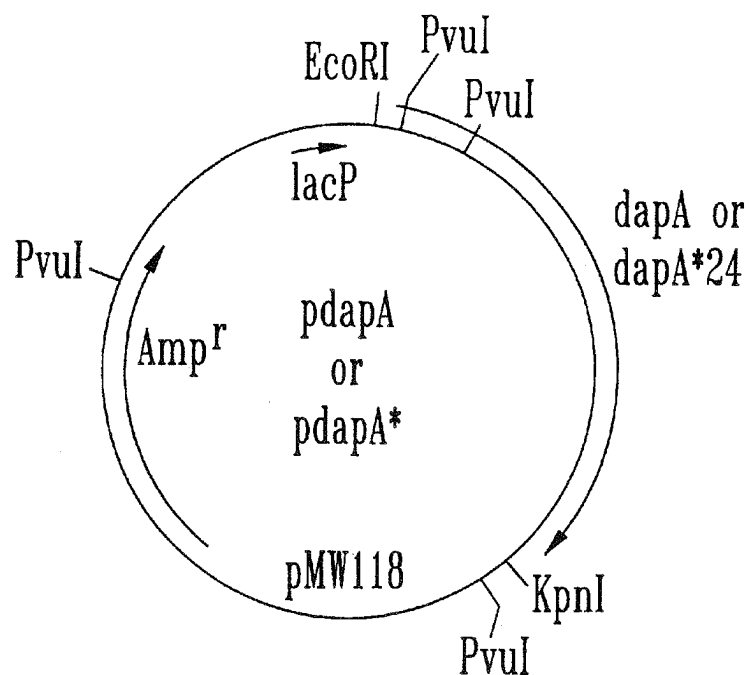
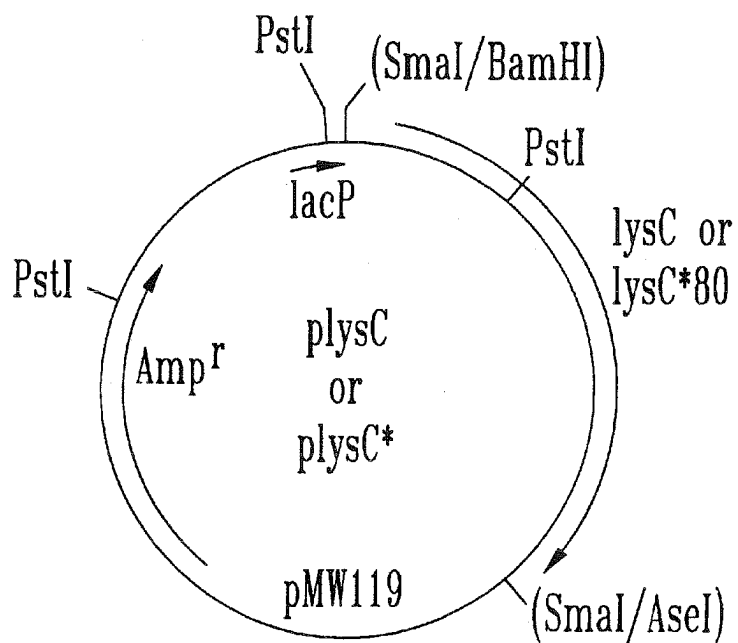
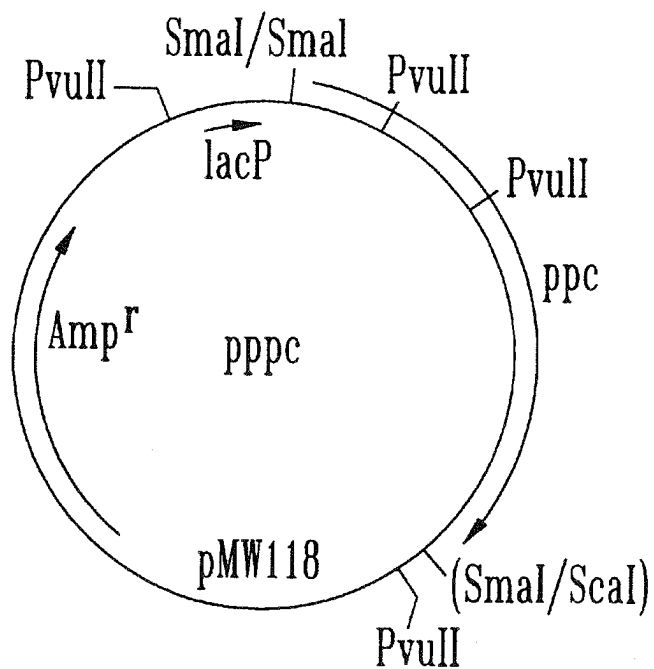
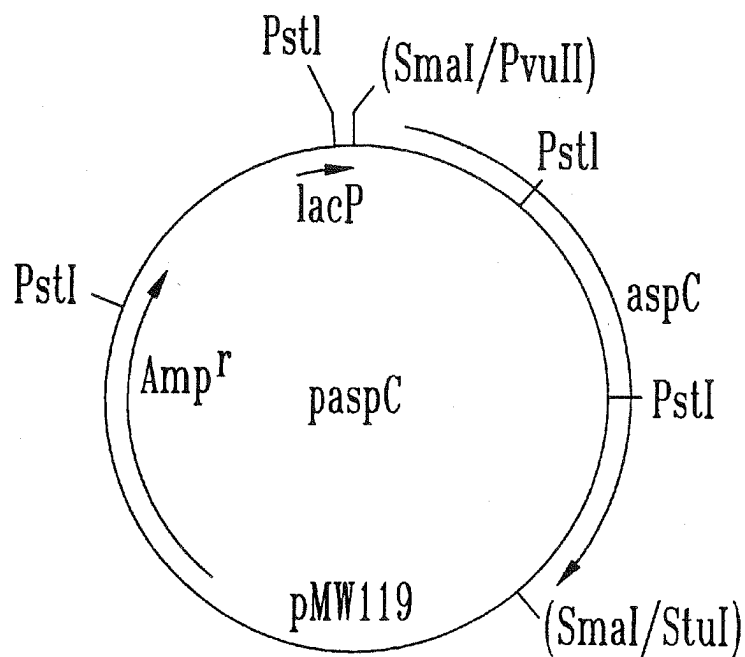


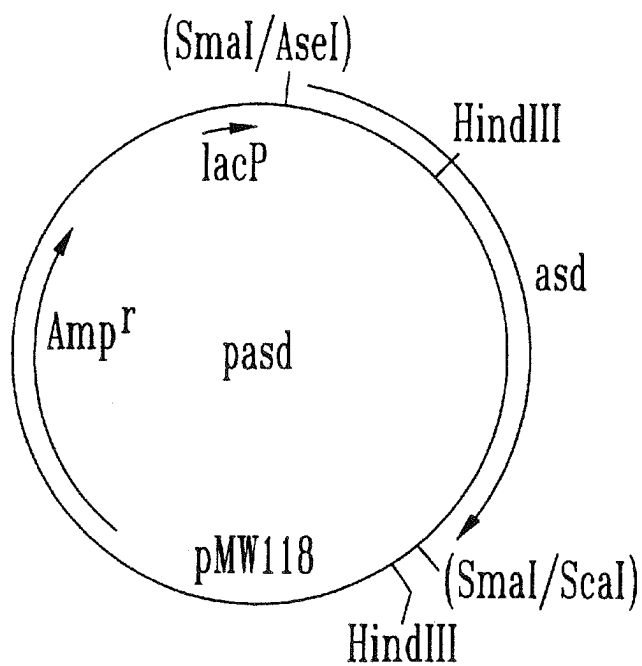
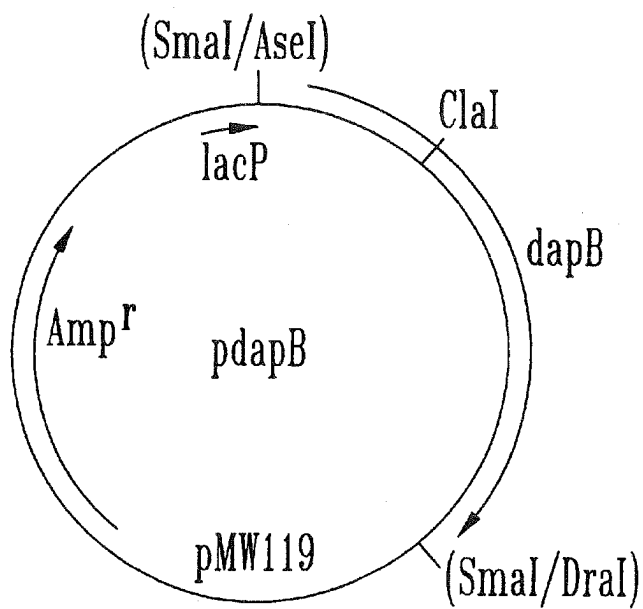
FIG. 7

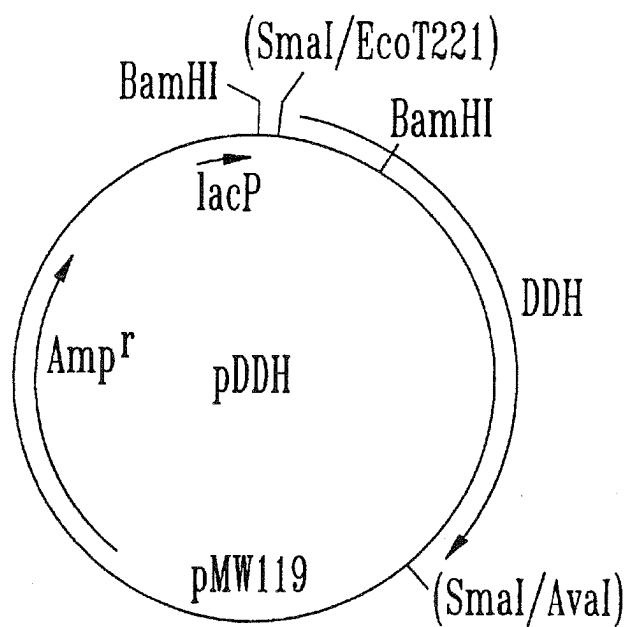
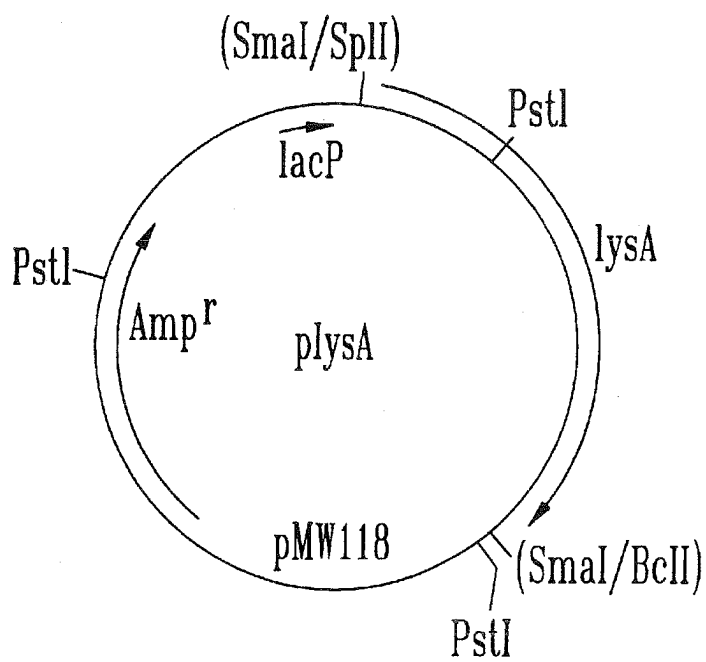
**FIG. 8**

**FIG. 9**

*FIG. 10**FIG. 11*

*FIG. 12**FIG. 13*

*FIG. 14**FIG. 15*

*FIG. 16**FIG. 17*

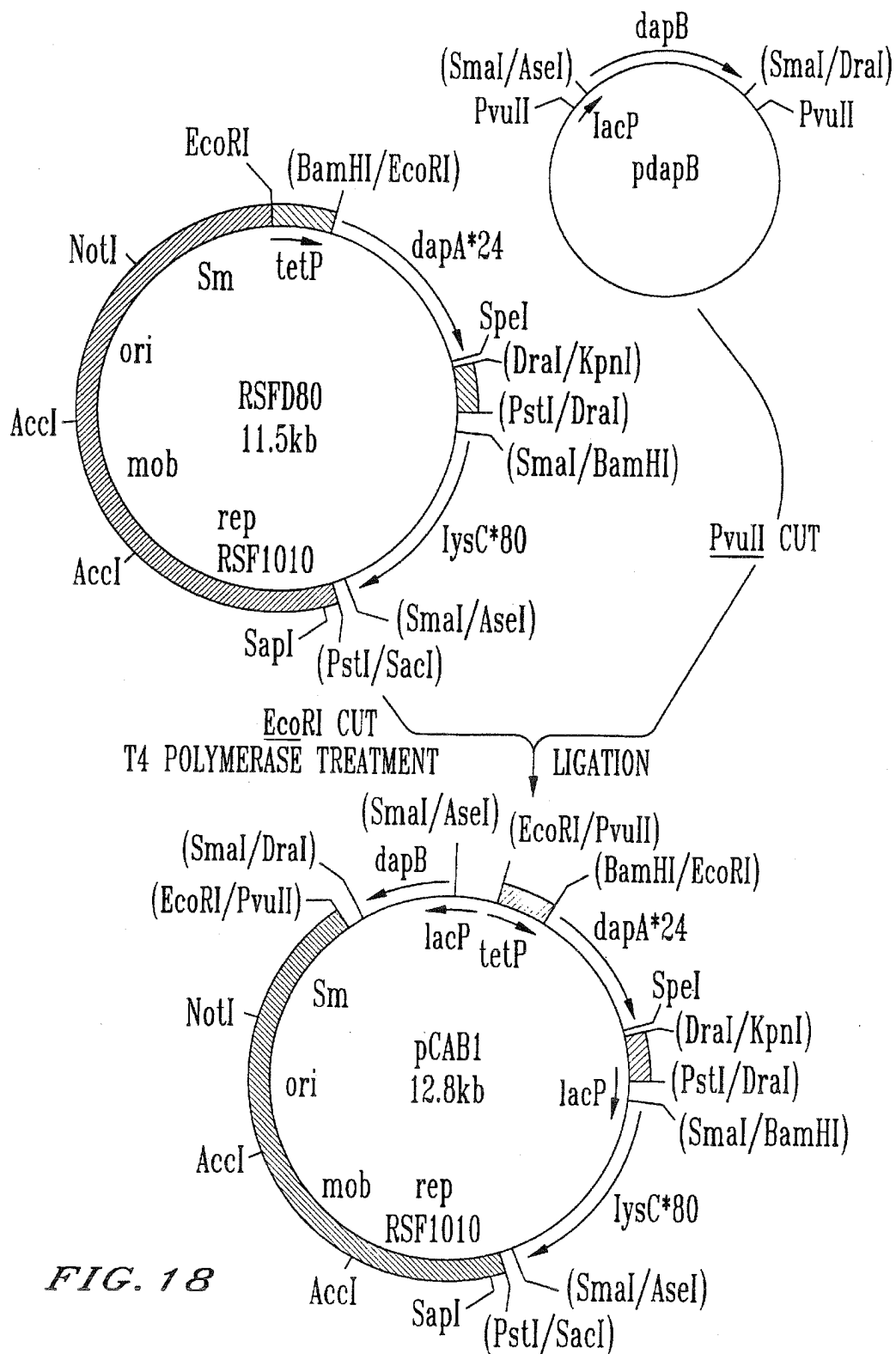


FIG. 18

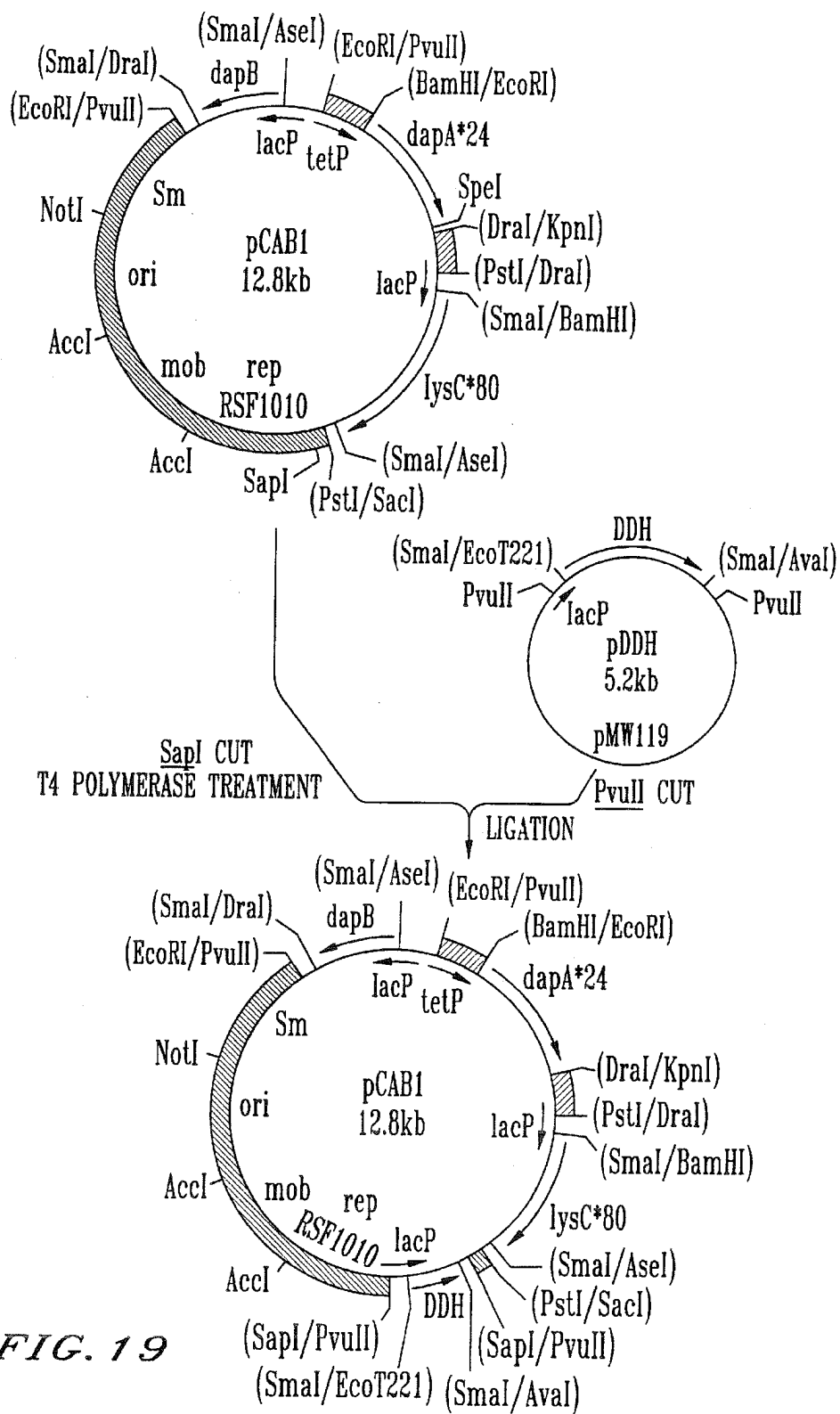


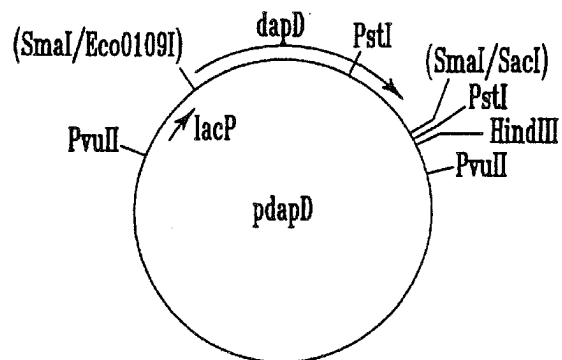
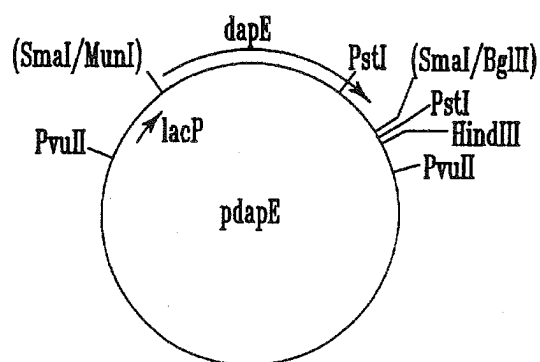
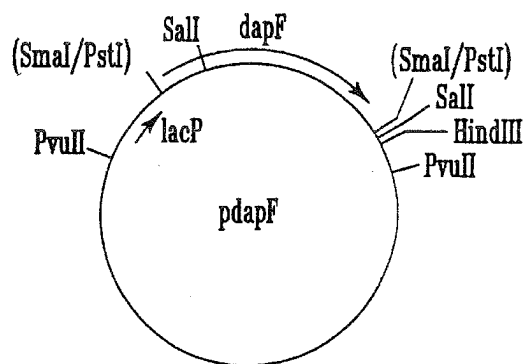
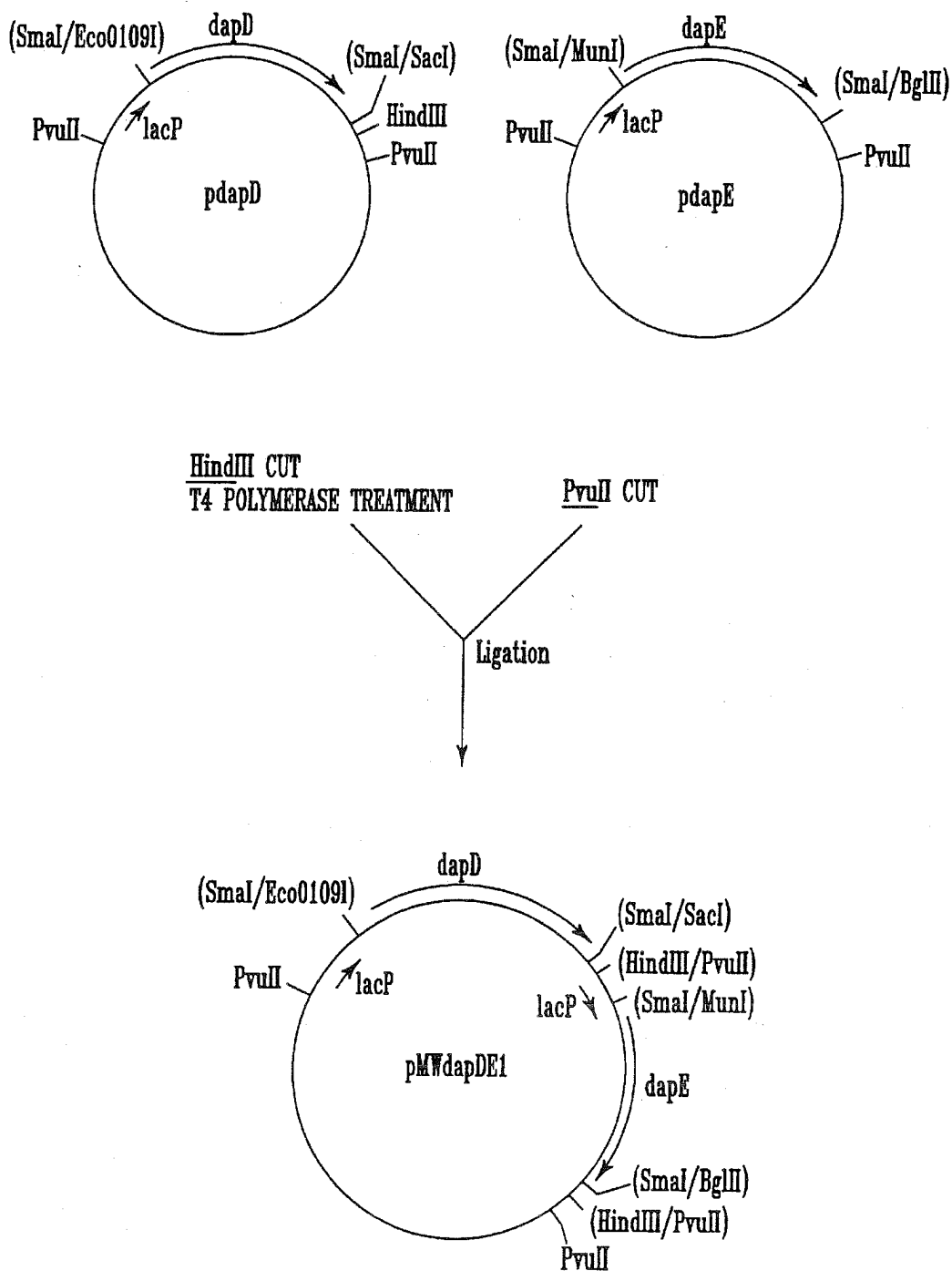
FIG. 20*FIG. 21**FIG. 22*

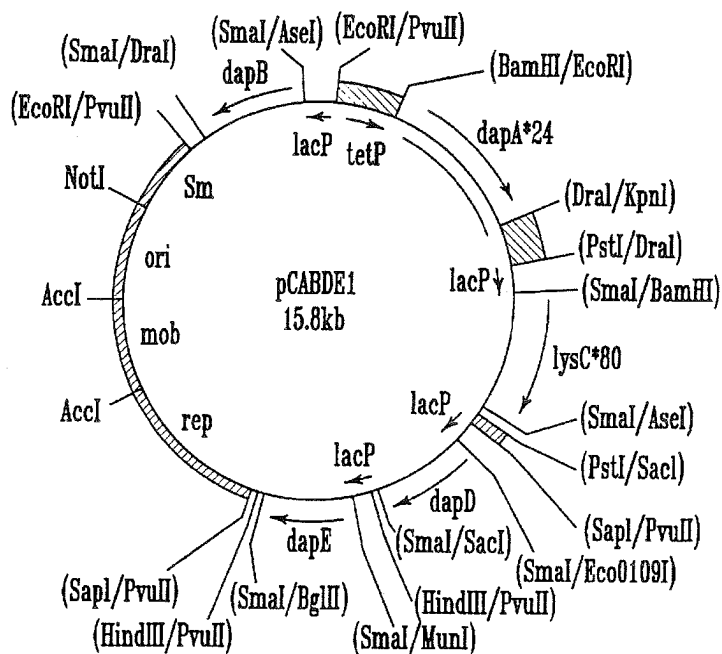
FIG. 23





Ligation

FIG. 24



METHOD OF PRODUCING L-LYSINE BY FERMENTATION

TECHNICAL FIELD

The present invention relates to microbial industry, and in particular relates to a method of producing L-lysine by fermentation, DNA's and microorganisms to be used for this production method.

BACKGROUND ART

In the prior art, when L-lysine is produced by a fermentative method, a microbial strain separated from the natural environment or an artificial mutant strain obtained from such a microbial strain is used in order to improve the productivity. A large number of artificial mutant strains producing L-lysine are known. Most of them are S-2-aminoethylcysteine (AEC) resistant mutant strains, and belong to the genus of *Brevibacterium*, *Corynebacterium*, *Bacillus* or *Escherichia*. Further, various techniques have been disclosed for increasing amino acid production, for example, by employing a transformant using recombinant DNA (U.S. Pat. No. 4,278,765).

With respect to those belonging to the genus *Escherichia*, for example, Japanese Patent Application Laid-open No. 56-18596, U.S. Pat. No. 4,346,170, and *Applied Microbiology and Biotechnology*, 15, 227-231 (1982) describe methods of producing L-lysine using a bacterial strain in which dihydrodipicolinate synthase (hereinafter sometimes abbreviated as "DDPS") is enhanced. However, DDPS used in these cases is a wild type, which suffers feedback inhibition by L-lysine. Thus sufficiently satisfactory L-lysine productivity has not been obtained. Incidentally, *Applied Microbiology and Biotechnology*, 15, 227-231 (1982) mentioned above is describes an L-lysine production of 3 g/l of L-lysine hydrochloride from 75 g/l of glucose, wherein a consumption coefficient (number of g of L-lysine produced from 1 g of sugar, or percentage thereof) is calculated to be 0.04, or 4%.

On the other hand, Korean Patent Publication No. 92-8382 describes a method of producing L-lysine using a bacterium belonging to *Escherichia* to which DDPS originating from a bacterium belonging to the genus *Corynebacterium*, which is known not to suffer feedback inhibition by L-lysine (consumption coefficient: 17%), is introduced. However, the upper limit temperature for growth of bacteria belonging to the genus *Corynebacterium* is lower than the upper limit temperature for growth of bacteria belonging to the genus *Escherichia* by about 10 degrees. Thus it seems that cultivation should be performed at a lowered cultivation temperature if DNA coding for DDPS originating from a bacterium belonging to the genus *Corynebacterium* is introduced into a bacterium belonging to the genus *Escherichia* in order to utilize it for L-lysine production. Therefore, it is anticipated that it is difficult to exhibit advantages possessed by the bacterium belonging to the genus *Escherichia* that the growth temperature is high, the growth speed is fast, and the L-lysine-producing speed is also fast. Generally, when a gene originating from a heterologous organism is expressed, there are occasionally caused decomposition of an expression product by protease and formation of an insoluble inclusion body, in which more difficulties are anticipated as compared with a case of expression of a homologous gene. Further, when DNA coding for DDPS originating from a bacterium belonging to the genus *Corynebacterium* is introduced into a bacterium belonging to the genus *Escherichia* to industrially produce

L-lysine, more strict regulation is obliged as compared with a case of use of a recombinant to which a homologous gene is introduced, in accordance with the recombinant DNA guideline.

By the way, the dihydrodipicolinate synthase (DDPS) is an enzyme for dehydrating and condensing aspartosemialdehyde and pyruvic acid to synthesize dihydrodipicolinic acid. This reaction is located at an entrance into a branch to proceed to an L-lysine biosynthesis system in biosynthesis of amino acids of the aspartic acid family. This enzyme is known to be in charge of an important regulatory site as aspartokinase is in bacteria belonging to the genus *Escherichia*.

DDPS is encoded by a gene called *dapA* in *E. coli* (*Escherichia coli*). The *dapA* has been cloned, and its nucleotide sequence has been also determined (Richaud, F. et al., *J. Bacteriol.*, 297 (1986)).

On the other hand, aspartokinase (hereinafter sometimes abbreviated as "AK") is an enzyme for catalyzing a reaction to convert aspartic acid into β -phosphoaspartic acid, which serves as a main regulatory enzyme in a biosynthesis system of amino acids of the aspartic acid family. AK of *E. coli* has three types (AKI, AKII, AKIII), two of which are complex enzymes with homoserine dehydrogenase (hereinafter sometimes abbreviated as "HD"). One of the complex enzymes is AKI-HDI encoded by a *thrA* gene, and the other is AKII-HDII encoded by a *metLM* gene. AKI is subjected to concerted suppression by threonine and isoleucine and inhibited by threonine, while AKII is suppressed by methionine.

On the contrary, it is known that only AKIII is a simple function enzyme, which is a product of a gene designated as *lysC*, and is subjected to suppression and feedback inhibition by L-lysine. The ratio of their intracellular activities is AKI:AKII:AKIII=about 5:1:4.

As described above, DDPS originating from bacteria belonging to the genus *Corynebacterium* is not subjected to feedback inhibition by L-lysine. However, when it is introduced into a bacterium belonging to the genus *Escherichia* to utilize it for L-lysine production, a problem arises in the cultivation temperature. It is expected that L-lysine can be efficiently produced by fermentation by using a bacterium belonging to the genus *Escherichia* if a mutant enzyme of DDPS or AKIII originating from a bacterium belonging to the genus *Escherichia*, which is not subjected to feedback inhibition by L-lysine, can be obtained. However, there is no preceding literature which describes such a mutant enzyme of DDPS, and although there is one report on a mutant enzyme of AKIII (Boy, E., et al., *J. Bacteriol.*, 112, 84 (1972)) no example has been known which suggests that such a mutant enzyme may improve productivity of L-lysine.

DISCLOSURE OF THE INVENTION

The present invention has been made taking the aforementioned viewpoints into consideration, an object of which is to obtain DDPS and AKIII originating from bacteria belonging to the genus *Escherichia* with sufficiently desensitized feedback inhibition by L-lysine, and provide a method of producing L-lysine by fermentation which is more improved than those in the prior art.

As a result of diligent and repeated investigation in order to achieve the object described above, the present inventors have succeeded in obtaining DNA coding for DDPS originating from a bacterium belonging to the genus *Escherichia* in which feedback inhibition by L-lysine is sufficiently

desensitized. The DNA coding for DDPS originating from *E. coli* in which feedback inhibition by L-lysine is sufficiently desensitized is sometimes referred to herein as mutant dapA or dapA*.

The inventors have further created a bacterium belonging to the genus *Escherichia* harboring mutant dapA and aspartokinase which is desensitized feedback inhibition by L-lysine. The DNA coding for aspartokinase originating from *E. coli* in which feedback inhibition by L-lysine is sufficiently desensitized is sometimes referred to herein as mutant lysC or lysC*.

The inventors have further created a bacterium belonging to the genus *Escherichia* harboring mutant dapA and mutant lysC. And it has been found that a considerable amount of L-lysine can be produced and accumulated in a culture by cultivating the aforementioned bacterium belonging to the genus *Escherichia* in a preferred medium.

The inventors have still further found that the productivity of L-lysine can be further improved by enhancing other genes in the L-lysine biosynthesis system of a bacterium belonging to the genus *Escherichia* harboring the mutant dapA and the mutant lysC.

Namely, the present invention lies in a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia* having mutation to desensitize feedback inhibition by L-lysine. The mutation to desensitize feedback inhibition by L-lysine is exemplified by mutation selected from the group consisting of mutation to replace a 81st alanine residue with a valine residue, mutation to replace a 118th histidine residue with a tyrosine residue, and mutation to replace the 81st alanine residue with the valine residue and replace the 118th histidine residue with the tyrosine residue, as counted from the N-terminal in an amino acid sequence of dihydrodipicolinate synthase defined in SEQ ID NO:4 in Sequence Listing.

The present invention further lies in a bacterium belonging to the genus *Escherichia* transformed by introducing, into its cells, a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia* having mutation to desensitize feedback inhibition by L-lysine. The mutation to desensitize feedback inhibition by L-lysine is exemplified by mutation to replace a 81st alanine residue with a valine residue, mutation to replace a 118th histidine residue with a tyrosine residue, and mutation to replace the 81st alanine residue with the valine residue and replace the 118th histidine residue with the tyrosine residue, as counted from the N-terminal in an amino acid sequence of dihydrodipicolinate synthase defined in SEQ ID NO:4 in Sequence Listing.

The present invention further lies in the aforementioned bacterium belonging to the genus *Escherichia* harboring an aspartokinase which is also desensitized feedback inhibition by L-lysine. A method to allow the bacterium belonging to the genus *Escherichia* to harbor the aspartokinase which is desensitized feedback inhibition by L-lysine is exemplified by a method for introducing, into its cells, a DNA coding for an aspartokinase III originating from a bacterium belonging to the genus *Escherichia* having mutation to desensitize feedback inhibition by L-lysine.

The mutation of the aspartokinase III to desensitize feedback inhibition by L-lysine is exemplified by mutation to replace a 323rd glycine residue with an aspartic acid residue, mutation to replace the 323rd glycine residue with the aspartic acid residue and replace a 408th glycine residue with an aspartic acid residue, mutation to replace a 34th arginine residue with a cysteine residue and replace the

323rd glycine residue with the aspartic acid residue, mutation to replace a 325th leucine residue with a phenylalanine residue, mutation to replace a 318th methionine residue with an isoleucine residue, mutation to replace the 318th methionine residue with the isoleucine residue and replace a 349th valine residue with a methionine residue, mutation to replace a 345th serine residue with a leucine residue, mutation to replace a 347th valine residue with a methionine residue, mutation to replace a 352nd threonine residue with an isoleucine residue, mutation to replace the 352nd threonine residue with the isoleucine residue and replace a 369th serine residue with a phenylalanine residue, mutation to replace a 164th glutamic acid residue with a lysine residue, and mutation to replace a 417th methionine residue with an isoleucine residue and replace a 419th cysteine residue with a tyrosine residue, as counted from the N-terminal in an amino acid sequence of aspartokinase III defined in SEQ ID NO:8 in Sequence Listing.

The DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia* having mutation to desensitize feedback inhibition by L-lysine, and the DNA coding for an aspartokinase III having mutation to desensitize feedback inhibition by L-lysine may be harbored on a chromosome of a bacterium belonging to the genus *Escherichia* respectively, or may be harbored in cells on an identical plasmid or separate plasmids. Further, it is also acceptable that one of the respective DNA's is harbored on a chromosome, and the other DNA is harbored on a plasmid.

The present invention still further lies in the aforementioned bacterium belonging to the genus *Escherichia* wherein a dihydrodipicolinate reductase gene is enhanced. The enhancement of the dihydrodipicolinate reductase gene can be achieved by transformation with a recombinant DNA constructed by ligating the dihydrodipicolinate reductase gene with a vector autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.

The present invention further lies in the aforementioned bacterium belonging to the genus *Escherichia* wherein an enhanced diaminopimelate dehydrogenase gene originating from coryneform bacteria such as *Brevibacterium lactofermentum* is introduced. The introduction of the enhanced diaminopimelate dehydrogenase gene originating from coryneform bacteria can be achieved by transformation with a recombinant DNA constructed by ligating the gene with a vector autonomously replicable in cells of bacteria belonging to the genus *Escherichia*. As coryneform bacteria, there may be exemplified wild type strains producing glutamic acid, and mutant strains thereof producing other amino acids, which belong to the genus *Corynebacterium* or the genus *Brevibacterium*. More concretely, *Brevibacterium flavum*, *Brevibacterium divaricatum*, *Corynebacterium glutamicum* and *Corynebacterium lilium* as well as *Brevibacterium lactofermentum* are exemplified as coryneform bacteria used for the present invention.

The present invention further lies in the bacterium belonging to the genus *Escherichia* wherein a tetrahydrodipicolinate succinylase gene and a succinyl diaminopimelate deacylase gene are enhanced instead of the aforementioned diaminopimelate dehydrogenase gene. The enhancement of these genes can be achieved by transformation with a single recombinant DNA or two recombinant DNA's constructed by ligating these genes with an identical vector or different vectors autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.

The present invention further provides a method of producing L-lysine comprising the steps of cultivating any of

the bacteria belonging to the genus *Escherichia* described above in an appropriate medium, producing and accumulating L-lysine in a culture thereof, and collecting L-lysine from the culture.

In this specification, DNA coding for DDPS or AKIII, or DNA containing a promoter in addition thereto is sometimes referred to as "DDPS gene" or "AKIII gene". Further, the mutant enzyme which is desensitized feedback inhibition by L-lysine, and DNA coding for it or DNA containing a promoter in addition to it are sometimes simply referred to as "mutant enzyme" and "mutant gene", respectively. Further, the phrase "feedback inhibition by L-lysine is desensitized" means that substantial desensitization of inhibition is sufficient, and complete desensitization is not necessary.

The present invention will be explained in detail below.
<1> DNA Coding for Mutant Dihydrodipicolinate Synthase (DDPS) of the Present Invention

The DNA coding for the mutant DDPS of the present invention has mutation to desensitize feedback inhibition by L-lysine of DDPS encoded in DNA coding for the wild type DDPS. DDPS is exemplified by those originating from bacteria belonging to the genus *Escherichia*, especially DDPS originating from *E. coli*. The mutation of DDPS to desensitize feedback inhibition by L-lysine is exemplified by:

- (1) mutation to replace a 81st alanine residue with a valine residue;
- (2) mutation to replace a 118th histidine residue with a tyrosine residue; and
- (3) mutation to replace the 81st alanine residue with the valine residue and replace the 118th histidine residue with the tyrosine residue; as counted from the N-terminal of DDPS in an amino acid sequence of DDPS defined in SEQ ID NO:4 in Sequence Listing.

The DNA coding for the wild type DDPS is not especially limited provided that it codes for DDPS originating from a bacterium belonging to the genus *Escherichia*, which is concretely exemplified by DNA coding for an amino acid sequence defined in SEQ ID NO:4, and is further concretely exemplified by a sequence represented by base numbers 272-1147 in a base sequence defined in SEQ ID NO:3. In these sequences, those having the mutation in nucleotide sequence to cause the replacement of amino acid residues described above are the DNA coding for the mutant DDPS of the present invention. Any codon corresponding to the replaced amino acid residue is available especially irrelevantly to its kind, provided that it codes for the identical amino acid residue. Further, it is postulated that possessed DDPS is slightly different in sequence depending on difference in bacterial species and bacterial strain, however, those having replacement, deletion or insertion of amino acid residue(s) at position(s) irrelevant to enzyme activity are also included in the mutant DDPS gene of the present invention.

A method for obtaining such a mutant gene is as follows. At first, a DNA containing a wild type DDPS gene or DDPS gene having another mutation is subjected to an in vitro mutation treatment, and a DNA after the mutation treatment is ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is introduced into a host microorganism to obtain transformants. When one which expresses a mutant DDPS is selected among the aforementioned transformants, such a transformant harbors a mutant gene. Alternatively, a DNA containing a wild type DDPS gene or DDPS gene having another mutation may be

ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is thereafter subjected to an in vitro mutation treatment, and a recombinant DNA after the mutation treatment is introduced into a host microorganism to obtain transformants. When one which expresses a mutant DDPS is selected among the aforementioned transformants, such a transformant also harbors a mutant gene.

It is also acceptable that a microorganism which produces a wild type enzyme is subjected to a mutation treatment to create a mutant strain which produces a mutant enzyme, and then a mutant gene is obtained from the mutant strain. Alternatively, a transformant to which a recombinant DNA ligated with a wild type gene is introduced may be subjected to a mutation treatment to create a mutant strain which produces a mutant enzyme. When a recombinant DNA is thereafter recovered from the mutant strain, a mutant gene is created on the aforementioned DNA.

The agent for performing the in vitro mutation treatment of DNA is exemplified by hydroxylamine and the like. Hydroxylamine is a chemical mutation treatment agent which causes mutation from cytosine to thymine by changing cytosine to N⁴-hydroxycytosine. Alternatively, when a microorganism itself is subjected to a mutation treatment, the treatment is performed by using ultraviolet light irradiation, or a mutagenic agent usually used for artificial mutation such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid.

No problem occurs when any one is used as a donor microorganism for DNA containing the wild type DDPS gene or DDPS gene having another mutation described above, provided that it is a microorganism belonging to the genus *Escherichia*. Concretely, it is possible to utilize those described in a book written by Neidhardt et al. (Neidhardt, F. C. et al., *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington D. C., 1208, table 1). For example, an *E. coli* JM109 strain and an MC1061 strain are exemplified. When a wild strain is used as a donor microorganism for DNA containing a DDPS gene, a DNA containing a wild type DDPS gene can be obtained.

(1) Preparation of Wild Type DDPS Gene

An example of preparation of DNA containing a DDPS gene will be described below. At first, *E. coli* having wild type dapA, for example, MC1061 strain, is cultivated to obtain a culture. When the microorganism described above is cultivated, cultivation may be performed in accordance with an ordinary solid culture method, however, cultivation is preferably performed by adopting a liquid culture method considering efficiency during collection of the bacterium. A medium may be used in which one or more nitrogen sources such as yeast extract, peptone, meat extract, corn steep liquor and exudate of soybean or wheat are added with one or more inorganic salts such as potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium sulfate, sodium chloride, magnesium chloride, ferric chloride, ferric sulfate or manganese sulfate, and further optionally and adequately added with sugar materials, vitamins and the like. It is appropriate that the initial pH of the medium is adjusted to 6-8. The cultivation is performed for 4-24 hours at 30-42° C., preferably at about 37° C. by means of deep culture with aeration and agitation, shaking culture or stationary culture or the like.

The culture thus obtained is centrifuged, for example, at 3,000 r.p.m. for 5 minutes to obtain a cell pellet of *E. coli* MC1061 strain. Chromosomal DNA can be obtained from the cell pellet by means of, for example, a method of Saito

and Miura (*Biochem. Biophys. Acta.*, 72, 619 (1963)), or a method of K. S. Kirby (*Biochem. J.*, 64, 405 (1956)).

In order to isolate the DDPS gene from the chromosomal DNA thus obtained, a chromosomal DNA library is prepared. At first, the chromosomal DNA is partially digested with a suitable restriction enzyme to obtain a mixture of various fragments. A wide variety of restriction enzymes can be used if the degree of cutting is controlled by the cutting reaction time and the like. For example, Sau3AI is allowed to react on the chromosomal DNA at a temperature not less than 30° C., preferably at 37° C. at an enzyme concentration of 1–10 units/ml for various periods of time (1 minute to 2 hours) to digest it.

Next, obtained DNA fragments are ligated with a vector DNA autonomously replicable in cells of bacteria belonging to the genus *Escherichia* to prepare recombinant DNA. Concretely, a restriction enzyme, which generates the terminal nucleotide sequence complement to that generated by the restriction enzyme Sau3AI used to cut the chromosomal DNA, for example, BmHI, is allowed to act on the vector DNA under a condition of a temperature not less than 30° C. and an enzyme concentration of 1–100 units/ml for not less than 1 hour, preferably for 1–3 hours to completely digest it, and cut and cleave it. Next, the chromosomal DNA fragment mixture obtained as described above is mixed with the cleaved and cut vector DNA, on which DNA ligase, preferably T4 DNA ligase is allowed to act under a condition of a temperature of 4–16° C. at an enzyme concentration of 1–100 units/ml for not less than 1 hour, preferably for 6–24 hours to obtain recombinant DNA.

The obtained recombinant DNA is used to transform a microorganism belonging to the genus *Escherichia*, for example, a DDPS deficient mutant strain such as an *Escherichia coli* K-12 strain, preferably a JE7627 strain (ponB704, dacB12, pfv⁺, tonA2, dapA, lysA, str, malA38, metB1, ilvH611, leuA371, proA3, lac-3, tsx-76) to prepare a chromosomal DNA library. The transformation can be performed, for example, by a method of D. M. Morrison (*Methods in Enzymology* 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159 (1970)). The JE7627 strain is available from National Institute of Genetics (Mishima-shi, Shizuoka-ken, Japan).

A bacterial strain having recombinant DNA of the DDPS gene is obtained from strains having increased DDPS activity or strains in which auxotrophy resulting from deficiency in DDPS gene is complemented, among the obtained chromosomal DNA library. For example, a DDPS deficient mutant strain requires diaminopimelic acid. Thus when the DDPS deficient mutant strain is used as a host, a DNA fragment containing the DDPS gene can be obtained by isolating a bacterial strain which becomes capable of growing on a medium containing no diaminopimelic acid, and recovering recombinant DNA from the bacterial strain.

Confirmation of the fact whether or not a candidate strain having recombinant DNA containing a DDPS gene actually harbors recombinant DNA in which the DDPS gene is cloned can be achieved by preparing a cellular extract from the candidate strain, and preparing a crude enzyme solution therefrom to confirm whether or not the DDPS activity has been increased. A procedure to measure the enzyme activity of DDPS can be performed by a method of Yugari et al. (Yugari, Y. and Gilvarg, C., *J. Biol. Chem.*, 240, 4710 (1962)).

Recombinant DNA in which DNA containing the DDPS gene is inserted into the vector DNA can be isolated from the

bacterial strain described above by means of, for example, a method of P. Guerry et al. (*J. Bacteriol.*, 116, 1064 (1973)) or a method of D. B. Clewell (*J. Bacteriol.*, 110, 667 (1972)).

Preparation of the wild type DDPS gene can be also performed by preparing chromosomal DNA from a strain having a DDPS gene on chromosome by means of a method of Saito and Miura or the like, and amplifying the DDPS gene by means of a polymerase chain reaction (PCR) method (see White, T. J. et al.; *Trends Genet.*, 5, 185 (1989)). DNA primers to be used for the amplification reaction are those complementary to both 3'-terminals of a double stranded DNA containing an entire region or a partial region of the DDPS gene. When only a partial region of the DDPS gene is amplified, it is necessary to use such DNA fragments as primers to perform screening of a DNA fragment containing the entire region from a chromosomal DNA library. When the entire region of the DDPS gene is amplified, a PCR reaction solution including DNA fragments containing the amplified DDPS gene is subjected to agarose gel electrophoresis, and then an aimed DNA fragment is extracted. Thus a DNA fragment containing the DDPS gene can be recovered.

The DNA primers may be adequately prepared on the basis of, for example, a sequence known in *E. coli* (Richaud, F. et al., *J. Bacteriol.*, 297 (1986)). Concretely, primers which can amplify a region comprising 1150 bases coding for the DDPS gene are preferable, and two species of primers defined in SEQ ID NO:1 and NO:2 are suitable. Synthesis of the primers can be performed by an ordinary method such as a phosphoamidite method (see *Tetrahedron Letters*, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems Inc.). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.), using Tag DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designated by the supplier.

With respect to the DDPS gene amplified by the PCR method, operations such as introduction of mutation into the DDPS gene become easy, when it is ligated with a vector DNA autonomously replicable in cells of bacteria belonging to the genus *Escherichia*, and introduced into cells of bacteria belonging to the genus *Escherichia*. The vector DNA to be used, the transformation method, and the confirmation method for the presence of the DDPS gene are the same as those in the aforementioned procedure.

(2) Introduction of Mutation Into DDPS Gene

The method for carrying out mutation such as replacement, insertion and deletion of amino acid residues is exemplified by a recombinant PCR method (Higuchi, R., 61, in *PCR Technology* (Erlich, H. A. Eds., Stockton press (1989))), and a site specific mutagenesis method (Kramer, W. and Frits, H. J., *Meth. in Enzymol.*, 154, 350 (1987); Kunkel T. A. et al., *Meth. in Enzymol.*, 154, 367 (1987)). Aimed mutation can be caused at an aimed site by using these methods.

Further, according to chemical synthesis of an aimed gene, it is possible to introduce mutation or random mutation into an aimed site.

Further, a method is available in which the DDPS gene on chromosome or plasmid is directly treated with hydroxylamine (Hashimoto, T. and Sekiguchi, M. *J. Bacteriol.*, 159, 1039 (1984)). Alternatively, it is acceptable to use a method in which a bacterium belonging to the genus *Escherichia* having the DDPS gene is irradiated by ultraviolet light, or a

method based on a treatment with a chemical agent such as N-methyl-N'-nitrosoguanidine or nitrous acid. According to these methods, mutation can be introduced randomly.

With respect to a selection method for the mutant gene, recombinant DNA comprising a DNA fragment containing the DDPS gene and vector DNA is at first directly subjected to a mutation treatment with hydroxylamine or the like, which is used to transform, for example, an *E. coli* W3110 strain. Next, transformed strains are cultivated on a minimal medium such as M9 containing S-2-aminoethylcysteine (AEC) as an analog of L-lysine. Strains harboring recombinant DNA containing the wild type DDPS gene cannot synthesize L-lysine and diaminopimelic acid (DAP) and are suppressed in growth because DDPS expressed from the recombinant DNA is inhibited by AEC. On the contrary, a strain harboring recombinant DNA containing the DDPS gene in which inhibition by L-lysine is desensitized has a mutant enzyme encoded by the DDPS gene in the aforementioned recombinant DNA which is not inhibited by AEC. Thus it should be capable of growth on the minimal medium in which AEC is added. This phenomenon can be utilized to select a strain which is resistant in growth to AEC as an analog of L-lysine, that is a strain harboring recombinant DNA containing a mutant DDPS gene in which inhibition is desensitized.

The mutant gene thus obtained may be introduced as a recombinant DNA into a suitable host microorganism, and expressed. Thus a microorganism can be obtained which harbors DDPS being desensitized feedback inhibition. The host is preferably a microorganism belonging to the genus *Escherichia*, for which *E. coli* is exemplified.

Alternatively, a mutant DDPS gene fragment may be taken out from the recombinant DNA, and inserted into another vector to make use. The vector DNA which can be used in the present invention is preferably plasmid vector DNA, for which there are exemplified pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219 and pMW218. Besides, vectors of phage DNA can be also utilized.

Further, in order to express the mutant DDPS gene efficiently, another promoter which works in microorganisms such as lac, trp and PL may be ligated upstream from a DNA sequence coding for the mutant DDPS, or a promoter contained in the DDPS gene may be used as it is, or after amplifying the promoter.

In addition, as described above, the mutant gene may be inserted into an autonomously replicable vector DNA, which is inserted into a host, and allowed to be harbored by the host as extrachromosomal DNA such as a plasmid. Alternatively, the mutant gene may be integrated into chromosome of a host microorganism by a method using transduction, transposon (Berg, D. E. and Berg, C. M., *Bio/Technol.*, 1, 417 (1983)), Mu phage (Japanese Patent Application Laid-open No. 2-109985) or homologous recombination (*Experiments in Molecular Genetics*, Cold Spring Harbor Lab. (1972)).

<2> DNA Coding for Mutant Aspartokinase III (AKIII) Used for the Present Invention

The DNA coding for mutant AKIII used for the present invention has mutation to desensitize feedback inhibition of encoded AKIII by L-lysine in DNA coding for wild type AKIII. The mutation to desensitize feedback inhibition of AKIII by L-lysine is exemplified by:

- (a) mutation to replace a 323rd glycine residue with an aspartic acid residue;
- (b) mutation to replace the 323rd glycine residue with the aspartic acid residue and replace a 408th glycine residue with an aspartic acid residue;

- (c) mutation to replace a 34th arginine residue with a cysteine residue and replace the 323rd glycine residue with the aspartic acid residue;
 - (d) mutation to replace a 325th leucine residue with a phenylalanine residue;
 - (e) mutation to replace a 318th methionine residue with an isoleucine residue;
 - (f) mutation to replace the 318th methionine residue with the isoleucine residue and replace a 349th valine residue with a methionine residue;
 - (g) mutation to replace a 345th serine residue with a leucine residue;
 - (h) mutation to replace a 347th valine residue with a methionine residue;
 - (i) mutation to replace a 352nd threonine residue with an isoleucine residue;
 - (j) mutation to replace the 352nd threonine residue with the isoleucine residue and replace a 369th serine residue with a phenylalanine residue;
 - (k) mutation to replace a 164th glutamic acid residue with a lysine residue; and
 - (l) mutation to replace a 417th methionine residue with an isoleucine residue and replace a 419th cysteine residue with a tyrosine residue;
- as counted from the N-terminal of AKIII in an amino acid sequence of AKIII defined in SEQ ID NO:8 in Sequence Listing.

The DNA coding for the wild type AKIII is not especially limited, for which DNA coding for AKIII originating from a bacterium belonging to the genus *Escherichia* such as *E. coli* is exemplified. Concretely, there are exemplified DNA coding for an amino acid sequence defined in SEQ ID NO:8, and a sequence represented by base numbers 584-1930 in a base sequence defined in SEQ ID NO:7. Incidentally, AKIII of *E. coli* is encoded by a lysC gene.

In these sequences, those which have mutation in base sequence to cause replacement of amino acid residues described above are DNA coding for the mutant AKIII of the present invention. Any codon corresponding to the replaced amino acid residue is available especially regardless of its kind, provided that it codes for the identical amino acid residue. Further, there are those in which amino acid sequences of possessed wild type AKIII are slightly different depending on difference in bacterial species and bacterial strains. Those having replacement, deletion or insertion of amino acid residue(s) at position(s) irrelevant to enzyme activity in such a manner are also included in the mutant AKIII gene of the present invention. For example, a base sequence of a wild type lysC gene obtained in Example 2 described below (SEQ ID NO:7) is different from an already published sequence of lysC of an *E. coli* K-12 JC411 strain at 6 sites (Cassan, M., Parsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261 1052 (1986)). Encoded amino acid residues are different at 2 sites of them (in lysC of the JC411 strain, a 58th glycine residue is replaced with a cysteine residue, and a 401st glycine residue is replaced with an alanine residue, as counted from the N-terminal in an amino acid sequence of lysC defined in SEQ ID NO:8). It is expected even for lysC having the same sequence as that of lysC of the *E. coli* K-12 JC411 strain that lysC having mutation in which feedback inhibition by L-lysine is desensitized is obtained if any of the aforementioned mutation of (a) to (1) is introduced.

A method for obtaining DNA coding for the mutant AKIII in which feedback inhibition by L-lysine is desensitized is as follows. At first, a DNA containing a wild type AKIII gene

or AKIII gene having another mutation is subjected to an in vitro mutation treatment, and a DNA after the mutation treatment is ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is introduced into a host microorganism to obtain transformants. When one which expresses a mutant AKIII is selected among the aforementioned transformants, such a transformant harbors a mutant gene. Alternatively, a DNA containing a wild type AKIII gene or AKIII gene having another mutation may be ligated with a vector DNA adapted to a host to obtain a recombinant DNA. The recombinant DNA is thereafter subjected to an in vitro mutation treatment, and a recombinant DNA after the mutation treatment is introduced into a host microorganism to obtain transformants. When one which expresses a mutant AKIII is selected among the aforementioned transformants, such a transformant also harbors a mutant gene.

Alternatively, it is also acceptable that a microorganism which produces a wild type enzyme is subjected to a mutation treatment to create a mutant strain which produces a mutant enzyme, and then a mutant gene is obtained from the mutant strain. The agent for performing a direct mutation treatment of DNA is exemplified by hydroxylamine and the like. Hydroxylamine is a chemical mutation treatment agent which causes mutation from cytosine to thymine by changing cytosine to N⁴-hydroxycytosine. Alternatively, when a microorganism itself is subjected to a mutation treatment, the treatment is performed by ultraviolet light irradiation, or using a mutating agent usually used for artificial mutation such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

Any one is used as a donor microorganism for DNA containing the wild type AKIII gene or AKIII gene having another mutation described above, provided that it is a microorganism belonging to the genus *Escherichia*. Concretely, it is possible to utilize those described in a book written by Neidhardt et al. (Neidhardt, F. C. et al., *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington D. C., 1208, table 1). For example, an *E. coli* JM109 strain and an MC1061 strain are exemplified. When the AKIII gene is obtained from these strains, preparation of chromosomal DNA, preparation of a chromosomal DNA library and the like may be performed in the same manner as the preparation of the DDPS gene described above. As the host to be used for preparation of the library, it is preferable to use a strain entirely deficient in AKI, II and III such as an *E. coli* GT3 strain (available from *E. coli* Genetic Stock Center (Connecticut, United States)).

From the obtained chromosomal DNA library, a bacterial strain having a recombinant DNA of the AKIII gene is obtained as a strain in which the AKIII activity is increased, or a strain in which auxotrophy is complemented. Cellular extracts are prepared from candidate strains, and crude enzyme solutions are prepared therefrom to confirm the AKIII activity. The measurement procedure for the AKIII enzyme activity may be performed in accordance with a method of Stadtman et al. (Stadtman, E. R., Cohen, G. N., LeBras, G., and Robichon-Szulmajster, H., *J. Biol. Chem.*, 236, 2033 (1961)).

For example, when a mutant strain completely deficient in AK is used as a host, a DNA fragment containing an AKIII gene can be obtained by isolating a transformed strain which becomes capable of growing on a medium not containing L-lysine, L-threonine, L-methionine and diaminopimelic acid, or on a medium not containing homoserine and diaminopimelic acid, and recovering recombinant DNA from the bacterial strain.

When the AKIII gene is amplified from chromosomal DNA by means of the PCR method, DNA primers to be used

for the PCR reaction can be properly prepared on the basis of, for example, a sequence known in *E. coli* (Cassan, M., Parsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261, 1052 (1986)). However, primers which can amplify a region comprising 1347 bases coding for lysC gene is suitable, and for example, two primers having sequences defined in SEQ ID NO:5 and NO:6 are suitable.

The method for carrying out mutation such as replacement, insertion and deletion of amino acid residue(s) on the AKIII gene obtained as described above is exemplified by the recombinant PCR method, the site specific mutagenesis method and the like, in the same manner as the mutation treatment of the DDPS gene described above.

Further, according to chemical synthesis of an aimed gene, it is possible to introduce mutation or random mutation into an aimed site.

Further, a method is available in which DNA of the AKIII gene on chromosome or extrachromosomal recombinant DNA is directly treated with hydroxylamine (Hashimoto, T. and Sekiguchi, M. *J. Bacteriol.*, 159, 1039 (1984)). Alternatively, it is acceptable to use a method in which a bacterium belonging to the genus *Escherichia* having an AKIII gene on chromosome or extrachromosomal recombinant DNA is irradiated by ultraviolet light, or a method to perform a treatment with a chemical agent such as N-methyl-N'-nitrosoguanidine or nitrous acid.

With respect to a selection method for the mutant AKIII gene, a strain completely deficient in AK, for example, an *E. coli* GT3 strain is at first transformed with a recombinant DNA containing an AKIII gene having been subjected to the mutation treatment. Next, transformed strains are cultivated on a minimal medium such as M9 containing a considerable amount of L-lysine. Strains harboring recombinant DNA containing a wild type AKIII gene cannot synthesize L-threonine, L-isoleucine, L-methionine and diaminopimelic acid (DAP) and are suppressed in growth because only one AK is inhibited by L-lysine. On the contrary, the strain harboring recombinant DNA containing the mutant AKIII gene in which inhibition by L-lysine is desensitized should be capable of growth on the minimal medium added with the considerable amount of L-lysine. This phenomenon can be utilized to select a strain which is resistant in growth to L-lysine or AEC as an analog of L-lysine, that is a strain harboring recombinant DNA containing a mutant AKIII gene in which inhibition is desensitized.

The mutant gene thus obtained may be introduced as a recombinant DNA into a suitable microorganism (host), and expressed. Thus a microorganism can be obtained which harbors AKIII being desensitized feedback inhibition.

The host is preferably a microorganism belonging to the genus *Escherichia*, for which *E. coli* is exemplified.

Alternatively, a mutant AKIII gene fragment may be taken out from the recombinant DNA, and inserted into another vector to make use. The vector DNA which can be used in the present invention is preferably plasmid vector DNA, for which there are exemplified pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219 and pMW218. Besides, vectors of phage DNA can be also utilized.

Further, in order to express the mutant AKIII gene efficiently, another promoter which works in microorganisms such as lac, trp and PL may be ligated upstream from a DNA sequence coding for the mutant AKIII, or a promoter contained in the AKIII gene may be used as it is, or after amplifying it.

In addition, as described above, the mutant gene may be inserted into an autonomously replicable vector DNA,

inserted into a host, and allowed to be harbored by the host as extrachromosomal DNA such as plasmid. Alternatively, the mutant gene may be integrated into chromosome of a host microorganism by a method using transduction, transposon (Berg, D. E. and Berg, C. M., *Bio/Technol.*, 1, 417 (1983)), Mu phage (Japanese Patent Application Laid-open No. 2-109985) or homologous recombination (*Experiments in Molecular Genetics*, Cold Spring Harbor Lab. (1972)).

<3> Production of L-lysine According to the Present Invention

L-lysine can be efficiently produced by cultivating, in a preferred medium, the bacterium transformed by introducing the mutant DDPS gene obtained as described above and allowed to harbor AK which is desensitized feedback inhibition by L-lysine, producing and accumulating L-lysine in a culture thereof, and collecting L-lysine from the culture. Namely, L-lysine can be efficiently produced by allowing the bacterium belonging to the genus *Escherichia* to harbor both of the mutant DDPS and the mutant AKIII.

The bacterium belonging to the genus *Escherichia* harboring AK which is desensitized feedback inhibition by L-lysine is exemplified by bacteria belonging to the genus *Escherichia* transformed by integrating, into chromosomal DNA, a DNA coding for AKIII having mutation to desensitize feedback inhibition by L-lysine, or bacteria belonging to the genus *Escherichia* transformed by introducing, into cells, a recombinant DNA constructed by ligating the DNA with a vector DNA autonomously replicable in cells of bacteria belonging to the genus *Escherichia*. Further, AK in which feedback inhibition by L-lysine is desensitized may be a wild type AK which does not suffer feedback inhibition by L-lysine, or one to which such a wild type AK gene is introduced into a bacterium belonging to the genus *Escherichia* in the same manner. Further, a mutant strain of a bacterium belonging to the genus *Escherichia*, which has become to produce a mutant AKIII by means of a mutation treatment of cells of a bacterium belonging to the genus *Escherichia*, is also acceptable.

On the other hand, in order to achieve transformation by introducing the mutant DDPS gene into a bacterium belonging to the genus *Escherichia*, the mutant DDPS gene may be integrated into chromosomal DNA to achieve transformation, or transformation may be achieved by introducing, into cells, a recombinant DNA constructed by ligating the mutant DDPS gene with a vector DNA autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.

When the both of the mutant DDPS gene and the mutant AKIII gene are introduced into a bacterium belonging to the genus *Escherichia*, the both mutant genes may be integrated into and harbored on chromosomal DNA of the bacterium belonging to the genus *Escherichia*, or they may be harbored on an identical plasmid or separated plasmids in cells as extrachromosomal DNA. When separated plasmids are used, it is preferable to use plasmids having a stable distribution mechanism to allow each of them to be compatibly harbored in the cell. Further, one of the mutant genes may be integrated into and harbored on chromosomal DNA, and the other mutant gene may be harbored on a plasmid in cells as extrachromosomal DNA, respectively. When the mutant DDPS gene and the mutant AKIII gene are introduced into a bacterium belonging to the genus *Escherichia*, any order of introduction of the both genes is acceptable.

The productivity of L-lysine can be further improved by enhancing a dihydrodipicolinate reductase gene of the bacterium belonging to the genus *Escherichia* in which the mutant DDPS gene and the mutant AKIII gene have been

introduced. The productivity of L-lysine can be still further improved by introducing a diaminopimelate dehydrogenase gene originating from a coryneform bacterium into the bacterium belonging to the genus *Escherichia* in which the dihydrodipicolinate reductase gene has been enhanced. This diaminopimelate dehydrogenase gene should be enhanced. Alternatively, the productivity of L-lysine can be also improved in a similar degree by enhancing tetrahydrodipicolinate succinylase gene and a succinyldiaminopimelate deacylase gene instead of the introduction of the diaminopimelate dehydrogenase.

The enhancement of gene herein refers to enhancement in activity of an enzyme as an expression product of the gene per a cell. Concretely, there may be exemplified enhancement in copy number of the gene in a cell, enhancement in expression amount per the gene by using a promoter having a high expression efficiency, and introduction of mutation to enhance enzyme activity into the gene. In order to enhance the copy number of a gene in a cell, the gene is inserted into a vector autonomously replicable in bacteria belonging to the genus *Escherichia*, and a bacterium belonging to the genus *Escherichia* may be transformed with this vector. This vector is preferably a multi-copy type plasmid. Alternatively, the copy number may be increased by amplifying DNA integrated into chromosomal DNA by using Mu phage or the like. With respect to the use of the plasmid, when plasmids are used for introduction of the mutant DDPS gene and the mutant AKIII gene, such plasmids having a stable distribution mechanism are preferably used in which these plasmids are stably harbored in a cell together. Any order of introduction of the genes is acceptable.

A mechanism will be explained below in which the productivity of L-lysine can be improved in a stepwise manner by successively enhancing genes of the L-lysine biosynthesis system as described above. A biosynthesis system comprising a plurality of reactions can be compared to a liquid flowing through a plurality of conduits having different thicknesses connected in serial. Herein each conduit corresponds to an individual enzyme, and the thickness of the conduit corresponds to an enzyme reaction velocity. In order to increase the amount of the liquid flowing through the conduits, it is effective to thicken the thinnest pipe. No effect can be expected even if a thick conduit is further thickened. In order to further increase the flow amount, the second thinnest conduit may be thickened. From such a viewpoint, the present inventors have tried to enhance the L-lysine biosynthesis system. For this purpose, as shown in Example 6 described below, the order of rate determining steps of the L-lysine biosynthesis system has been elucidated by introducing, into *E. coli*, genes of the L-lysine biosynthesis system originating from *E. coli* in a stepwise manner. In this elucidation, four genes of dapC succinyldiaminopimelate transaminase dapD (tetrahydrodipicolinate succinylase gene) dapE (succinyldiaminopimelate deacylase gene), and dapF (diaminopimelate epimerase gene) located downstream in the biosynthesis pathway were replaced with a gene DDH coding for DDH (diaminopimelate dehydrogenase) of *Brevibacterium lactofermentum* capable of catalyzing reactions participated by these gene products by itself. Namely, introduced genes for enzymes of the L-lysine biosynthesis system and the enzymes encoded by them are as follows:

ppc: phosphoenolpyruvate carboxylase
aspC: aspartate aminotransferase
lysC: aspartokinase III
lysC*: inhibition-desensitized aspartokinase III

asd: aspartate semialdehyde dehydrogenase
dapA: dihydrodipicolinate synthase
dapA*: inhibition-desensitized dihydrodipicolinate synthase
dapB: dihydrodipicolinate reductase
DDH: diaminopimelate dehydrogenase (originating from *Brevibacterium lactofermentum*)
lysA: diaminopimelate decarboxylase

As a result of individual introduction of each of the genes into *E. coli*, production of L-lysine was found in strains in which lysC*, dapA or dapA* was introduced, and a dapA*-introduced strain showed the highest L-lysine productivity. According to the result, it was found that a reaction catalyzed by dapA was the first rate determining step. Next, when each of the genes of the L-lysine biosynthesis system was introduced into the dapA*-introduced strain, lysC* had the largest effect on the improvement in L-lysine productivity. Thus it was found that a reaction catalyzed by lysC was the second rate determining step. In the same manner, it was found that a reaction catalyzed by dapB was the third rate determining step, and a reaction catalyzed by DDH was the fourth rate determining step. Further, as a result of investigation on rate determining steps among reactions catalyzed by dapC, dapD, dapE and dapF replaced with DDH, it was found that dapD and dapE concerned rate determining.

A method for obtaining the genes of the L-lysine biosynthesis system of *E. coli* and the DDH gene of *Brevibacterium lactofermentum* will be exemplified below.

The ppc gene can be obtained from a plasmid pS2 (Sabe, H. et al., *Gene*, 31, 279 (1984)) or pT2 having this gene. A DNA fragment containing the ppc gene is obtained by cutting pS2 with AatII and AflIII. A DNA fragment having the ppc gene is also obtained by cutting pT2 with SmaI and ScaI. An *E. coli* F15 strain (AJ12873) harboring pT2 is internationally deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) under a deposition number of FERM BP-4732 based on the Budapest Treaty.

The aspc gene is obtained from a plasmid pLF4 (Inokuchi, K. et al., *Nucleic Acids Res.*, 10, 6957 (1982)) having this gene. A DNA fragment having the aspc gene is obtained by cutting pLF4 with PvuII and StuI.

The asd gene is obtained from a plasmid pAD20 (Haziza, C. et al., *EMBO*, 1, 379 (1982)) having this gene. A DNA fragment having the asd gene is obtained by cutting pAD20 with AseI and ClaI.

The dapB gene is obtained by amplifying chromosomal DNA of *E. coli* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:9, NO:10) prepared on the basis of a nucleotide sequence of a known dapB gene (Bouvier, J. et al., *J. Biol. Chem.*, 259, 14829 (1984)).

The DDH gene is obtained by amplifying chromosomal DNA of *Brevibacterium lactofermentum* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:11, NO:12) prepared on the basis of a known nucleotide sequence of a DDH gene of *Corynebacterium glutamicum* (Ishino, S. et al., *Nucleic Acids Res.*, 15, 3917 (1987)).

The lysA gene is obtained by amplifying chromosomal DNA of *E. coli* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:13, NO:14) prepared on the basis of a nucleotide sequence of a known lysA gene (Stragier, P. et al., *J. Mol. Biol.*, 168, 321 (1983)).

The dapD gene is obtained by amplifying chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:15, NO:16) prepared on the basis of a nucleotide sequence of a known dapD gene (Richaud, C. et al., *J. Biol. Chem.*, 259, 14824 (1984)).

The dapE gene is obtained by amplifying *E. coli* DNA by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:17, NO:18) prepared on the basis of a nucleotide sequence of a known dapE gene (Bouvier, J. et al., *J. Bacteriol.*, 174, 5265 (1992)).

The dapF gene is obtained by amplifying chromosomal DNA of *E. coli* by means of the PCR method by using two species of oligonucleotide primers (for example, SEQ ID NO:19, NO:20) prepared on the basis of a nucleotide sequence of a known dapF gene (Richaud, C. et al., *Nucleic Acids Res.*, 16, 10367 (1988)).

In the present invention, any bacterium belonging to the genus *Escherichia* is available for the use as a host provided that a promoter of the mutant DDPS gene, the mutant AKIII gene or another gene of the L-lysine biosynthesis system, or another promoter for expressing these genes functions in its cells, and a replication origin of a vector DNA to be used for introduction functions in its cells to be capable of replication when the mutant DDPS gene, the mutant AKIII gene or another gene of the L-lysine biosynthesis system is introduced into a plasmid as extrachromosomal DNA.

For example, there may be exemplified L-lysine-producing *E. coli*, concretely a mutant strain having resistance to L-lysine analogs. The lysine analog is such one which inhibits proliferation of bacteria belonging to the genus *Escherichia*, but the suppression is entirely or partially desensitized if L-lysine co-exists in a medium. For example, there are oxalysine, lysine hydroxamate, AEC, γ -methyllysine, α -chlorocapro lactam and the like. Mutant strains having resistance to these lysine analogs are obtained by applying an ordinary artificial mutation operation to microorganisms belonging to the genus *Escherichia*. The bacterial strain to be used for L-lysine production is concretely exemplified by *Escherichia coli* AJ11442 (deposited as FERM BP-1543 and NRRL B-12185; see Japanese Patent Application Laid-open No. 56-18596 or U.S. Pat. No. 4,346, 170). In aspartokinase of the microorganisms described above, feedback inhibition by L-lysine is desensitized.

Besides, for example, L-threonine-producing microorganisms are exemplified, because inhibition of their aspartokinase by L-lysine is generally desensitized also in the L-threonine-producing microorganisms. As an L-threonine-producing bacterium belonging to *E. coli*, a B-3996 strain has the highest producibility known at present. The B-3996 strain is deposited in Research Institute for Genetics and Industrial Microorganism Breeding under a registration number of RIA 1867.

The medium to be used for cultivation of the transformant harboring the mutant gene according to the present invention is an ordinary medium containing a carbon source, a nitrogen source, organic ions and optionally other organic components.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose, or starch hydrolysate; alcohols such as glycerol or sorbitol; or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin B₁ and L-isoleucine or yeast extract to be contained in

appropriate amounts as organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

Cultivation is preferably carried out under an aerobic condition for 16–72 hours. The cultivation temperature is controlled at 25° C. to 45° C., and pH is controlled at 5–8 during cultivation. Inorganic or organic, acidic or alkaline substances as well as ammonia gas or the like can be used for pH adjustment.

Collection of L-lysine from a fermented liquor is usually carried out by combining an ion exchange resin method, a precipitation method and other known methods.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows preparation steps for pdapA1 and pdapA2.

FIG. 2 shows inhibition by L-lysine for wild type and mutant DDPS's.

FIG. 3 shows preparation steps for a plasmid pdapAS824 having a double mutation type dapA* gene.

FIG. 4 shows preparation steps for pLYSC1 and pLYSC2.

FIG. 5 shows an appearance ratio and a mutation ratio of transformants after a hydroxylamine treatment.

FIG. 6 shows inhibition by L-lysine for wild type and mutant AKIII's.

FIG. 7 shows preparation steps for a plasmid RSF24P originating from RSF1010 having dapA*24.

FIG. 8 shows preparation steps for a plasmid pLLC*80.

FIG. 9 shows preparation steps for a plasmid RSFD80 originating from RSF1010 having dapA*24 and lysC*80.

FIG. 10 shows structures of plasmids pdapA and pdapA* having dapA or dapA*.

FIG. 11 shows structures of plasmids pLYSC and pLYSC* having lysC or lysC*80.

FIG. 12 shows a structure of a plasmid pppc having ppc.

FIG. 13 shows a structure of a plasmid paspc having aspc.

FIG. 14 shows a structure of a plasmid pasd having asd.

FIG. 15 shows a structure of a plasmid pdapB having dapB.

FIG. 16 shows a structure of a plasmid pDDH having DDH.

FIG. 17 shows a structure of a plasmid pLYSA having lysA.

FIG. 18 shows preparation steps for a plasmid pCAB1 originating from RSF1010 having dapA*24, lysC*80 and dapB.

FIG. 19 shows preparation steps for a plasmid pCABD2 originating from RSF1010 having dapA*24, lysC*80, dapB and DDH.

FIG. 20 shows a structure of a plasmid pdapD having dapD.

FIG. 21 shows a structure of a plasmid pdapE having dapE.

FIG. 22 shows a structure of a plasmid pdapF having dapF.

FIG. 23 shows preparation steps for a plasmid pMW-dapDE1 having dapD and dapE.

FIG. 24 shows preparation steps for a plasmid pCABDE1 having dapA*24, lysC*80, dapB, dapD and dapE.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be more concretely explained below with reference to Examples.

EXAMPLE 1

Preparation of Mutant DDPS Gene

<1> Cloning of Wild Type dapA Gene

A nucleotide sequence of a dapA gene of *E. coli* has been already reported (Richaud, F. et al., *J. Bacteriol.*, 297 (1986)), and it is known that its open reading frame (ORF) comprises 876 base pairs, and codes for a protein comprising 292 amino acid residues. Since it is unknown how this dapA gene is regulated, a region containing only an SD sequence and ORF except for a promoter region was amplified by using the PCR method and cloned.

Total genomic DNA of an *E. coli* K-12 MC1061 strain was extracted in accordance with a method of Saito and Miura (*Biochem. Biophys. Acta.*, 72, 619 (1963)). Two species of primers having sequences shown in SEQ ID NO:1 and NO:2 were prepared, which were used to perform the PCR reaction in accordance with a method of Erlich et al. (*PCR Technology*, Stockton press (1989)), and target DNA was amplified. Obtained DNA was inserted into a commercially available cloning vector pCR1000 for PCR fragments (purchased from Invitrogen, Ltd., (California, the United States)) as it was. pCR1000 contains a lacZ promoter (Placz), and is sold in a state of being cut at a site downstream from the lacZ promoter. When a recombinant DNA obtained by ligating a PCR fragment between both cut termini of pCR1000 is introduced into *E. coli*, the PCR fragment is transcribed under control of the lacZ promoter. Upon ligation of the PCR fragment with PCR1000, two species of plasmids were obtained, which were pdapA1 as a plasmid ligated in a normal orientation and pdapA2 as a plasmid ligated in a reversed orientation, for the direction of transcription of dapA with respect to the direction of transcription by the lacZ promoter (FIG. 1).

When these plasmids were introduced into *E. coli* JE7627 which is a strain deficient in DDPS, strains with the introduced plasmids is complemented auxotrophy for diaminopimelic acid of the host JE7627. Thus it was confirmed that DNA fragments inserted into the both plasmids contain the gene dapA coding for active DDPS.

A transformed strain obtained by introducing pdapA1 into a wild type *E. coli* W3110 strain (available from National Institute of Genetics (Mishima-shi, Shizuoka-ken, Japan)) was designated as W3110/pdapA1, and a transformed strain obtained by introducing pdapA2 into the *E. coli* W3110 strain was designated as W3110/pdapA2, respectively. These two transformed strains were cultivated respectively in a minimal medium M9 having the following composition added with AEC as an analog of lysine. The W3110 strain with no introduced plasmid was also cultivated in the same medium as a control. These two transformed strains and the W3110 strain having no plasmid were suppressed in growth by AEC, however, their growth inhibition was recovered by addition of L-lysine.

(Minimal medium M9)

A:	(20 × M9)	
	Na ₂ HPO ₄ ·12H ₂ O	303 g/L
	KH ₂ PO ₄	60 g/L
	NaCl	10 g/L
	NH ₄ Cl	20 g/L

B: 1 M MgSO₄

C: 50% Glucose

D: 1 g/L Thiamine

A, B, C and D described above were separately sterilized, and mixed in a ratio of A:B:C:D: water=5:0.1:0.1:95.

<2> Preparation of Mutant DDPS Gene (dapA*)

It was assumed that a strain harboring a plasmid containing dapA* coding for DDPS with desensitized inhibition by L-lysine could grow on a minimal medium M9 added with a considerable amount of AEC. A strain harboring a plasmid containing dapA* was selected by their growth resistance to AEC.

In order to efficiently obtain dapA*, dapA's on pdapA1 and pdapA2 prepared in <1> were subjected to a mutation treatment.

(1-2-1) Investigation on Selection Condition for Strain Harboring Plasmid Containing dapA*

The W3110/pdapA1 strain and the W3110/pdapA2 strain obtained as described above were cultivated on M9 agar plate media containing various concentrations of AEC, respectively. Growth inhibitory concentrations by AEC were examined, and a selection condition was investigated for a strain harboring a plasmid containing dapA*.

Growth of the transformants on the M9 media containing AEC at various concentrations is shown in Table 1. In this table, +indicates growth of transformant, and—indicates no growth.

TABLE 1

AEC concentration (mM)	W3110/pdapA1	W3110/pdapA2
250	—	—
125	—	—
60	—	—
30	—	—
15	+	—
8	+	+
4	+	+
2	+	+

The direction of transcription of the dapA gene on pdapA1 coincides with the direction of transcription by the lacZ promoter (FIG. 1). Thus it was found that the dapA gene on pdapA1 provided resistance to AEC at considerably high concentrations even when dapA remained as a wild type because its expression amount was amplified by the lacZ promoter, while the dapA gene on pdapA2 had a smaller expression amount and provided inhibition in growth by AEC at lower concentrations because the direction of transcription was in the reversed direction with respect to the lacZ promoter, and a promoter of dapA's own was also deficient (the growth was suppressed in an allotment of addition of 30 mM in the case of the W3110/pdapA1 strain, and of 15 mM in the case of the W3110/pdapA2 strain). It was confirmed that the growth inhibition was eliminated by simultaneous addition of L-lysine.

Therefore, pdapA2 was used as an object for introduction of mutation. A medium prepared by adding 60 mM of AEC to the minimal medium M9 was used for selection of a strain harboring a plasmid containing dapA*. This medium is referred to as "selection medium" in Example 1 below.

(1-2-2) In Vitro Mutation Treatment for pdapA2 with Hydroxylamine

An in vitro mutation treatment method in which plasmids are directly treated with hydroxylamine was used for introduction of mutation into the pdapA2 plasmid.

2 μ g of DNA was treated at 75° C. for 1–4 hours in 0.4 M hydroxylamine (0.1 M KH_2PO_4 -1 mM EDTA (pH 6.0): 100 μ l, 1 M hydroxylamine-1 mM EDTA (pH 6.0): 80 μ l, DNA: 2 μ g, total: 200 μ l by filling up with water). DNA after the treatment was purified with glass powder, introduced into *E. coli* W3110, and spread on a complete medium (L-broth: 1%

Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar), and colonies were formed. They were replicated onto the selection medium described in (1-2-1), and those which formed colonies on the selection medium were selected. Candidates of mutant plasmids in a total of 36 strains were obtained after two times of experiments.

The candidate strains of 36 strains in total thus obtained were spotted on the selection medium again, and AEC resistance was confirmed.

(1-2-3) Isolation of dapA* Gene and Investigation on dapA* Product

Mutant pdapA2's were recovered from the 36 strains described above. A dapA-deficient strain, JE7627 was transformed with them and the wild type pdapA2, respectively. A cell-free extract was prepared from each of the transformed strains, and the enzyme activity of DDPS was measured.

The cell-free extract (crude enzyme solution) was prepared as follows. A transformed strain was cultivated in a 2xTY medium (1.6% Bacto trypton, 1% Yeast extract, 0.5% NaCl), and collected at an optical density at 660 nm (OD_{660}) of about 0.8. A cell pellet was washed with 0.85% NaCl under a condition of 0° C., and suspended in 20 mM potassium phosphate buffer (pH 7.5) containing 400 mM KCl. The cells were ruptured by sonication (0° C., 200 W, 10 minutes). A ruptured cell solution was centrifuged at 33 krpm for 1 hour under a condition of 0° C. to obtain a supernatant to which ammonium sulfate was added to give 80% saturation to be stored at 0° C. overnight followed by centrifugation. A pellet was dissolved in 20 mM potassium phosphate buffer (pH 7.5)-400 mM KCl.

The enzyme activity of DDPS was measured in accordance with a method of Yûgari et al. (Yûgari, Y. and Gilvarg, C., *J. Biol. Chem.*, 240, 4710 (1962)). Namely, the absorbance of a reaction solution having the following composition was measured at 37° C. with a spectrophotometer at a wavelength of 270 nm in a time-dependent manner. And generated dihydridipicolinate was measured. Sodium pyruvate was removed from the reaction system to be used as a blank.

(Composition of Reaction Solution)

50 mM imidazole-HCl pH 7.4
20 mM L-aspartate semialdehyde
20 mM sodium pyruvate
enzyme solution
water (balance)
total 1.0 ml

Various concentrations of L-lysine were added to the enzyme reaction solution during measurement of the enzyme activity of DDPS, and the degree of inhibition by L-lysine was examined. As shown in FIG. 2, the wild type DDPS suffered inhibition by L-lysine. Mutant plasmids originating from the transformed strains having DDPS difficult to suffer inhibition by L-lysine as compared with the wild type were three species among the 36 species of the candidate plasmids. They were designated as pdapAS8, pdapAS9 and pdapAS24, respectively. According to following determination of nucleotide sequences, it was revealed that pdapAS8 and pdapAS9 had the same mutation.

The degree of desensitization of inhibition by L-lysine was varied in the three species of mutant DDPS encoded by pdapAS8, pdapAS9 and pdapAS24, however, the inhibition by L-lysine was desensitized in all of the three species. Although the specific activity of the enzyme might be affected by growth situations of cells and preparation of samples, it was found to be lowered a little in any case as compared with the wild type. However, it was judged that no substantial problem would be caused by them as a material for breeding.

(1-2-4) Determination of Nucleotide Sequence of Mutant dapA Gene

Nucleotide sequences of the mutant dapA genes were determined in accordance with an ordinary method by using a DNA sequencer ABI Model 373A (produced by Applied Biosystems Inc.). As a result, it was revealed that 487th C was changed to T in pdapAS8 and pdapAS9, and 597th C was changed to T in pdapAS24 on a sequence of the wild type dapA gene shown in SEQ ID NO:3. Therefore, it was revealed that a 81st alanine residue was changed to a valine residue in DDPS encoded by pdapAS8 and pdapAS9, and a 118th histidine residue was changed to a tyrosine residue in DDPS encoded by pdapAS24 in an amino acid sequence of DDPS shown in SEQ ID NO:4.

(1-2-5) Preparation of dapA Having Double Mutation

Two species of the mutant dapA genes were obtained as described above. In order to verify whether or not desensitization of inhibition works additively for these mutations, a plasmid containing mutant dapA having both of the two mutations was prepared. A procedure of preparation is as shown in FIG. 3. An obtained plasmid having double mutation was designated as pdapAS824.

EXAMPLE 2

Preparation of Mutant AKIII Gene

<1> Cloning of Wild Type lysC Gene

A nucleotide sequence of an AKIII gene (lysC) of *E. coli* has been already reported (Cassan, M., Parsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261, 1052 (1986)), and it is known that its open reading frame (ORF) comprises 1347 base pairs, and codes for a protein comprising 449 amino acid residues. An operator is present in this gene, and is subjected to suppression by L-lysine. Thus in order to remove the operator region, a region containing only an SD sequence and ORF was amplified by using the PCR method and cloned.

Total genomic DNA of an *E. coli* K-12 MC1061 strain was prepared in accordance with a method of Saito and Miura (*Biochem. Biophys. Acta.*, 72, 619 (1963)). Two species of primers having sequences shown in SEQ ID NO:5 and NO:6 were prepared, which were used to perform the PCR reaction in accordance with a method of Erlich et al. (*PCR Technology*, Stockton press (1989)), and the lysC gene was amplified. Obtained DNA was digested with BamHI and AseI, then blunt-ended, and inserted into a SmaI site of a multi-copy vector, pUC18. This SmaI site is located at a downstream side from a lacZ promoter existing in the vector, and when recombinant DNA obtained by inserting a DNA fragment into the SmaI site of pUC18 is introduced into *E. coli*, the inserted DNA fragment is transcribed by means of

site of pUC18, two species of plasmids were obtained, which were pLYSC1 as a plasmid inserted in a reversed orientation and pLYSC2 as a plasmid inserted in a normal orientation, for the direction of transcription of lysC with respect to the direction of transcription by the lacZ promoter (FIG. 4).

When these plasmids were used to transform *E. coli* GT3 (thrA1016b, metLM1005, lysC1004) as a completely deficient strain for AKI, II, III, auxotrophy of GT3 for homoserine and diaminopimelic acid was complemented. Thus it was confirmed that DNA fragments inserted into the both plasmids contain the gene lysC coding for active AKIII.

A transformed strain obtained by introducing pLYSC1 into the AK completely deficient strain, *E. coli* GT3 was designated as GT3/pLYSC1, and a transformed strain obtained by introducing pLYSC2 into the *E. coli* GT3 was designated as GT3/pLYSC2. A considerable amount of L-lysine was added to the minimal medium M9, and the GT3/pLYSC1 strain and the GT3/pLYSC2 strain were cultivated, respectively. Both of the GT3/pLYSC1 strain and the GT3/pLYSC2 strain harbor plasmids containing the wild type lysC, in which AKIII encoded by lysC on the plasmids is a sole AK. The wild type AKIII as the sole AK is inhibited by L-lysine in the presence of a considerable amount of L-lysine. Thus the both strains could not synthesize L-threonine, L-isoleucine, L-methionine and diaminopimelic acid (DAP), and were suppressed in growth.

<2> Preparation of Mutant AKIII Gene (lysC*)

It was assumed that a strain harboring a plasmid containing lysC* coding for AK with desensitized inhibition by L-lysine could grow on a minimal medium M9 added with a considerable amount of L-lysine. A strain harboring a plasmid containing lysC* was selected by selecting strains with their growth resistant to L-lysine or AEC as an analog of L-lysine.

In order to efficiently obtain lysC*, lysC's on pLYSC1 and pLYSC2 prepared in <1> were subjected to a mutation treatment.

(2-2-1) Investigation on Selection Condition for Strain Harboring Plasmid Containing lysC*

The GT3/pLYSC1 strain and the GT3/pLYSC2 strain were cultivated on M9 agar plate media containing various concentrations of L-lysine or AEC, respectively. Growth inhibitory concentrations by L-lysine or AEC were examined, and a selection condition was investigated for a strain harboring a plasmid containing lysC*.

Growth of the transformants on the M9 media containing L-lysine or AEC at various concentrations is shown in Table 2. In this table, + indicates growth of transformant, ± indicates a little growth, and - indicates no growth.

TABLE 2

	Growth and L-lysine concentration											
	0	0.2	0.4	0.8	1.5	3	6	12	25	50	100	200 (mM)
GT3/pLYSC1	+	-	-	-	-	-	-	-	-	-	-	-
GT3/pLYSC2	+	+	+	+	+	+	+	+	+	+	+	-
	Growth and AEC concentration											
	0	0.2	0.4	0.8	1.5	3	6	12	25	50 (mM)		
GT3/pLYSC1	+	-	-	-	-	-	-	-	-	-	-	-
GT3/pLYSC2	+	±	±	±	±	±	±	±	±	±	±	±

read-through transcription under the control by the lacZ promoter. Upon insertion of the PCR fragment into the SmaI

The direction of transcription of the lysC gene on pLYSC2 coincides with the direction of transcription by the

lacZ promoter (FIG. 4). Thus it was found that the *lysC* gene on pLYSC2 provided resistance to L-lysine and AEC at considerably high concentrations even when *lysC* remained as a wild type because its expression amount was amplified by the lacZ promoter, while the *lysC* gene on pLYSC1 had a smaller expression amount and provided inhibition in growth by L-lysine and AEC at lower concentrations because the direction of transcription was in the reversed direction with respect to the lacZ promoter, and a promoter of itself was also deficient (the growth was not suppressed up to an allotment of addition of 100 mM for L-lysine and up to an allotment of addition of 3 mM for AEC in the case of the GT3/pLYSC2 strain, while the growth was completely suppressed in an allotment of addition of 0.2 mM for both L-lysine and AEC in the case of GT3/pLYSC1 strain). It was confirmed that the growth inhibition was eliminated by simultaneous addition of homoserine and diaminopimelic acid.

Therefore, pLYSC1 was used for experiments of introduction of mutation. A medium prepared by adding 10 mM of L-lysine or 0.2 mM of AEC to the minimal medium M9 was used for selection of plasmid-harboring strains containing *lysC**. This medium is referred to as "selection medium" in Example 2 below.

(2-2-2) In Vitro Mutation Treatment for pLYSC1 with Hydroxylamine

Two kinds of methods were used for introduction of mutation into the pLYSC1 plasmid, which were an in vitro mutation treatment method in which plasmids are directly treated with hydroxylamine, and an additional in vivo mutation treatment method in which a cell harboring a plasmid is treated with nitrosoguanidine (NTG) followed by extraction of the plasmid in order to provide diversity of mutation, namely expecting mutation other than the mutation from cytosine to thymine with hydroxylamine.

(In Vitro Mutation Treatment with Hydroxylamine)

2 μ g of DNA was treated under a condition of 75° C. for 1-4 hours in 0.4 M hydroxylamine (0.1 M KH_2PO_4 -1 mM EDTA (pH 6.0): 100 μ l, 1 M hydroxylamine-1 mM EDTA (pH 6.0): 80 μ l, DNA: 2 μ g, total: 200 μ l by filling up with water). DNA after the treatment was purified with glass powder, introduced into an AK completely deficient strain, an *E. coli* GT3 strain, and spread on a complete medium (L-broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar), and colonies were formed. They were replicated onto the selection medium described in (2-2-1), and strains capable of growth on the selection medium were selected as candidate strains. The appearance ratio of transformants and the mutation ratio were found to proceed as shown in FIG. 5. Mutant strains were obtained by a treatment for 4 hours at a considerably high ratio of 0.5-0.8%.

(In Vivo Mutation Treatment with NTG)

pLYSC1 was introduced into *E. coli* MC1061, and an NTG treatment was performed with a whole cell. The cell after the treatment was cultivated overnight to fix mutation, and then a plasmid was extracted and introduced into *E. coli* GT3. Namely, the transformed strain was cultivated in a 2 \times TY medium (1.6% Bacto trypton, 1% Yeast extract, 0.5% NaCl), collected at an OD_{660} of about 0.3, washed with a TM buffer described below, then suspended in an NTG solution (prepared by dissolving NTG at a concentration of 0.2 mg/ml in TM buffer), and treated at 37° C. for 0-90 minutes. The cell was washed with TM buffer and 2 \times TY medium, and then mutation was fixed by cultivation in 2 \times TY medium overnight. Subsequently plasmid DNA was extracted from the cell, and introduced into an *E. coli* GT3 strain. Screening of candidate strains was performed in the

same manner as in the in vitro mutation, and mutants of lysine resistance (*Lys^R*) and AEC resistance (*AEC^R*) were obtained.

(TM buffer)	
Tris	50 mM
Maleic acid	50 mM
(NH_4) ₂ SO ₄	1 g/L
MgSO ₄ ·7H ₂ O	0.1 g/L
Ca(NO ₃) ₂	5 mg/L
FeSO ₄ ·7H ₂ O	0.25 mg/L

pH was adjusted to 6.0 with NaOH.

Total 180 strains of candidate strains obtained as described above (hydroxylamine treatment: 48 strains, NTG treatment: 132 strains) were spotted on the selection medium again, and AEC and L-lysine resistances were confirmed to obtain 153 strains. Taking a notice of difference in amino acid accumulation pattern in the medium, these 153 strains were divided into 14 groups, and the AK activity was measured after selecting representative strains of each of the groups. There was no large difference in AK activity between the mutant strains obtained by the hydroxylamine treatment and the mutant strains obtained by the NTG treatment. Thus the following experiments were performed without distinguishing them.

(2-2-3) Isolation of *lysC** Gene and Investigation on *lysC** Product

No. 24, No. 43, No. 48, No. 60, No. 80, No. 117, No. 126, No. 149, No. 150, No. 156, No. 158, No. 167, No. 169 and No. 172 were selected as representative strains of the aforementioned 14 groups. Mutant plasmids derived from pLYSC1 were recovered from each of them, and designated as pLYSC1*24, pLYSC1*43, pLYSC1*48, pLYSC1*60, pLYSC1*80, pLYSC1*117, pLYSC1*126, pLYSC1*149, pLYSC1*150, pLYSC1*156, pLYSC1*158, pLYSC1*167, pLYSC1*169 and pLYSC1*172, respectively. An AK completely deficient strain GT3 was transformed with them and the wild type pLYSC1. A cell-free extract was prepared from each of transformed strains, and the enzyme activity of AKIII was measured.

The cell-free extract (crude enzyme solution) was prepared as follows. A transformed strain was cultivated in a 2 \times TY medium, and collected at an OD_{660} of about 0.8. Cells were washed with 0.02 M KH_2PO_4 (pH 6.75)-0.03 M β -mercaptoethanol under a condition of 0° C., and the cells were ruptured by sonication (0° C., 100 W, 30 minutes \times 4). A ruptured cell solution was centrifuged at 33 krpm for 1 hour under a condition of 0° C. to obtain a supernatant, to which ammonium sulfate was added to give 80% saturation. After centrifugation, a pellet was dissolved in 0.02 M KH_2PO_4 (pH 6.75)-0.03 M β -mercaptoethanol, and stored at 0° C. overnight.

The enzyme activity of AKIII was measured in accordance with a method of Stadtman et al. (Stadtman, E. R., Cohen, G. N., LeBras, G., and Robichon-Szulmajster, H., *J. Biol. Chem.*, 236, 2033 (1961)). Namely, a reaction solution having the following composition was incubated at 27° C. for 45 minutes, and an FeCl_3 solution (2.8 N HCl 0.4 ml +12% TCA 0.4 ml +5% FeCl_3 , 6H₂O/0.1 N HCl 0.7 ml) was added to develop a color, which was centrifuged followed by measurement of absorbance of a supernatant at 540 nm. The activity was indicated by an amount of hydroxamic acid generated per minute (1 U=1 μ mol/min). The molar absorption coefficient was 600. Potassium aspartate was removed from the reaction solution to be used as a blank.

(Composition of Reaction Solution)

Reaction mixture *1	0.3 ml
Hydroxylamine solution *2	0.2 ml
0.1 M Potassium aspartate (pH 7.0)	0.1 ml
Enzyme solution	
Water	(balance)
	total 1.0 ml

*1: 1 M Tris-HCl (pH 8.1) 9 ml + 0.3 M MgSO₄ 0.5 ml + 0.2 M ATP (pH 7.0) 5 ml

*2: 8 M Hydroxylamine solution was neutralized just before use with KOH.

Various concentrations of L-lysine were added to the enzyme reaction solution for measurement of the enzyme activity of AK, and the degree of inhibition by L-lysine was examined. Results are shown in FIG. 6 and Table 3. The wild type and Nos. 24, 43, 48, 60, 80, 117 and 126 are shown in FIG. 6A. Nos. 149, 150, 156, 158, 167, 169 and 172 are shown in FIG. 6B.

As shown in these results, the wild type AKIII strongly suffered inhibition by L-lysine, which was inhibited by 50% at about 0.45 mM of L-lysine, and inhibited by about 100% at 5 mM. On the contrary, the mutant AKIII's obtained this time had various degrees of desensitization, however, inhibition by L-lysine was desensitized in all of 14 species. Especially in the case of Nos. 24, 80, 117, 169 and 172, inhibition was scarcely observed even at 100 mM of L-lysine, and they had 50%-inhibitory-concentrations which were not less than 200 times as compared with that of the wild type. The specific activity per total protein, which might be affected by growth situations of cell and preparation of samples, was equal to or more than that of the wild type in almost all cases, in which there was little problem of decrease in activity due to the introduction of mutation (Table 3). According to this fact, it was postulated that an active center of AKIII was independent from a regulatory site by L-lysine with each other. In Table 3, the inhibition desensitization degree (%) refers to an AK activity in the presence of 100 mM of L-lysine with respect to an AK activity in the absence of L-lysine in the reaction solution. The heat stability (%) refers to a ratio of activity maintenance after a treatment at 55° C. for 1.5 hour.

TABLE 3

	Specific activity (U/mg protein)	Degree of desensitization of inhibition (%) ^{*1}	Heat stability (%) ^{*2}
Wild type	0.0247	0	18
No. 117	0.0069	120	0
No. 24	0.0218	100	30
No. 80	0.0244	99	36
No. 172	0.0189	97	0
No. 169	0.0128	96	2
No. 150	0.0062	77	25
No. 126	0.0250	61	39
No. 149	0.0256	59	9
No. 167	0.0083	43	45
No. 48	0.0228	38	42
No. 60	0.0144	35	9
No. 158	0.0224	22	42
No. 156	0.0101	18	2
No. 43	0.0212	17	0

*1: AK activity (%) in the presence of 100 mM of L-lysine with respect to AK activity in the absence of L-lysine

*2: ratio of activity maintenance (%) after treatment at 55° C. for 1.5 hour

Subsequently, the heat stability of the mutant enzymes was examined. When it is intended that an enzyme is improved to increase its activity, it is important that a created enzyme is maintained stably in cells. Measurement in vitro has some problems because of the difference in intracellular

and extracellular protease activities and the influence of buffers for in vitro storage of enzymes. However, for convenience, the heat stability of the mutant AKIII's was investigated in vitro as one parameter.

Judging from results of investigation on the inactivation temperature of AKIII under various conditions, the ratio of activity maintenance after a treatment at 55° C. for 90 minutes was measured. As shown in Table 3, half the enzymes were rather more excellent than the wild type. Generally, a mutant enzyme is often unstable as compared with a wild type. However, some of the mutant AKIII's obtained this time were superior to the wild type in stability, and many of them seemed to be fairly useful in practical use for L-lysine production.

(2-2-4) Determination of Base Sequence of Wild Type lysC and Mutant lysC

A nucleotide sequence of the wild type lysC gene obtained this time was determined in accordance with an ordinary method by using a DNA sequencer ABI Model 373A (produced by Applied Biosystems Inc.) (SEQ ID NO:7). As a result, differences were found in six sites (two places at the amino acid level) from an already published sequence of lysC of an *E. coli* K-12 JC411 strain (Cassan, M., Rarsot, C., Cohen, G. N., and Patte, J. C., *J. Biol. Chem.*, 261, 1052 (1986)). It is speculated that the difference in six sites is due to the difference in bacterial strain used.

In the same manner, base sequences were determined for each of lysC*'s existing on the 14 species of mutant pLYSC1's, and mutation points were clarified. Results are shown in Table 4. In this table, indications in parentheses show mutations of amino acid residues based on mutations of nucleotides. Types of mutations were 12 kinds because two sets (No. 4 and No. 167, No. 24 and No. 80) had exactly the same mutation types among the 14 species. With respect to mutation types, Nos. 149, 150, 156, 158, 167, 169 and 172 were obtained by the hydroxylamine treatment, and Nos. 24, 43, 48, 60, 80, 117 and 126 were obtained by the NTG treatment. However, as for the pattern of mutation, any of them resided in mutation from cytosine to thymine, or mutation from guanine to adenine on a coding strand due to mutation from cytosine to thymine on a noncoding strand.

TABLE 4

Determination of mutation points of lysC*

lysC* mutation type	Mutagen	Mutation point (amino acid change)
No. 126	N	GGT→GA*T (²²³ Gly→Asp)
No. 43	N	GGT→GA*T (²²³ Gly→Asp)
		GGC→GA*C (⁴⁰⁸ Gly→Asp)
No. 149	H	CGT→T*GT (³⁴ Arg→Cys)
		GGT→GA*T (²²³ Gly→Asp)
No. 48/167	N/H	CTC→T*TC (³²⁵ Leu→Phe)
No. 150	H	ATG→ATA* (³¹⁸ Met→Ile)
No. 172	H	⁷⁷⁵ C→T (silent)
		ATG→ATA* (³¹⁸ Met→Ile)
		GTG→A*TG (³⁴⁹ Val→Met)
No. 117	N	TCA→TT*A (³⁴⁵ Ser→Leu)
No. 158	H	GTG→A*TG (³⁴⁷ Val→Met)
No. 24/80	N/N	ACC→AT*C (³⁵² Thr→Ile)
No. 169	H	⁹²³ C→T (silent)
		ACC→AT*C (³⁵² Thr→Ile)
		TCT→TT*T (³⁶⁰ Ser→Phe)
No. 60	N	⁸⁵⁹ G→A (silent)
		GAA→A*AA (¹⁶⁴ Glu→Lys)
No. 156	H	ATG→ATA* (⁴¹⁷ Met→Ile)
		TGT→TA*T (⁴¹⁹ Cys→Tyr)
		²⁰¹⁴ C→T (silent)

*: H; hydroxylamine treatment, N; NTG treatment

EXAMPLE 3

Fermentation Production of L-lysine with Strain Being Introduced daDA*

In order to produce L-lysine by using *E. coli*, as indicated in Japanese Patent Application Laid-open No. 56-18596, U.S. Pat. No. 4,346,170 and *Applied Microbiology and Biotechnology*, 15, 227-231 (1982), it is considered to be essential that a host for enhancing DDPS has an aspartokinase which is changed not to suffer inhibition by L-lysine. L-threonine-producing bacteria may be exemplified as such a strain. As for L-threonine-producing *E. coli*, a B-3996 strain has the highest productivity among those known at present. Thus the B-3996 strain was used as a host for evaluating dapA*. The B-3996 strain harbors pVIC40 extra-chromosomally as a sole plasmid. Details are described in Japanese Patent Laid-open No. 3-501682 (PCT). This microorganism is deposited in Research Institute for Genetics and Industrial Microorganism Breeding under a registration No. of RIA 1867.

On the other hand, dapA* contained in pdapAS24 (in which the 118th histidine residue replaced with a tyrosine residue) was selected as dapA* to be introduced into *E. coli*, judging from the degree of desensitization of inhibition and the specific activity of the enzyme. At first, in order to increase the expression amount of dapA* and increase stability of the plasmid, mutant dapA* having existed on pdapAS24 (hereinafter referred to as "dapA*24") was ligated at the downstream from a promoter of a tetracycline resistance gene of pVIC40, and RSF24P was obtained as shown in FIG. 7.

A strain obtained by introducing the plasmid RSF24P into an *E. coli* JM109 strain was designated as AJ12395, which is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology on October 28, 1993, as accession number of FERM P-13935, and transferred from the original deposition to international deposition based on Budapest Treaty on Nov. 1, 1994, and has been deposited as accession number of FERM BP-4858. Strains harboring pdapAS8 and pdapAS9 were not deposited. However, all of the mutation points of dapA* on each of the plasmids have been clarified as described above. Thus it is easy for those skilled in the art that the plasmid is recovered from the aforementioned deposited bacterium by using a method of Maniatis et al. (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1.21 (1989)), and a target gene is obtained by using a site-directed mutagenesis method (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 15.63 (1989)).

pVIC40 was deleted from the B-3996 strain in accordance with an ordinary method, and a B-399 strain was obtained as a strain having no plasmid. The plasmid RSF24P was introduced into the B-399 strain in accordance with an ordinary method, and B-399/RSF24P was obtained. The L-lysine productivity of B-399/RSF24P was evaluated.

On the other hand, RSFP was constructed as a control plasmid. Namely, a large fragment was selected from digest of pVIC40 double-digested with BamHI and DraI as shown in FIG. 7, and it was blunt-ended with DNA polymerase Klenow fragment. The blunt-ended large fragment was self-ligated to obtain the plasmid RSFP. RSFP was introduced into the B-399 strain in accordance with an ordinary method, and B-399/RSFP was obtained. The L-lysine productivity was also evaluated for B-399/RSFP.

The cultivation was performed at an agitation of 114-116 rpm under a condition of a cultivation period of 48 hours and a temperature of 37° C. by using the following medium. Results are shown in Table 5.

(Medium for L-lysine Production)

A:	(NH ₄) ₂ SO ₄	16 g/L
	KH ₂ PO ₄	1 g/L
	MgSO ₄ ·7H ₂ O	1 g/L
	FeSO ₄ ·7H ₂ O	0.01 g/L
	NaSO ₄ ·5H ₂ O	0.01 g/L
	Yeast Ext. (Difco)	2 g/L
	L-methionine	0.5 g/L
	L-threonine	0.1 g/L
	L-isoleucine	0.05 g/L
	pH is adjusted to 7.0 with KOH to be autoclave at 115° C. for 10 minutes.	(16/20 volume)
B:	20% Glucose (autoclave at 115° C. for 10 minutes)	(4/20 volume)
C:	Pharmacopoeial CaCO ₃ (heat-sterilized in dry state at 180° C. for 2 days)	(30 g/L)

A and B are mixed in the ratio of A:B=4:1, 30 g of C is added to 1 L of the mixture and dissolved, and antibiotics (streptomycin: 100 µg/ml, kanamycin: 5 µg/ml) are added.

TABLE 5

Bacterial strain	Production amount of L-lysine hydrochloride
B-399/RSF24P	4.1 g/L
B-399/RSFP	0 g/L

EXAMPLE 4

Fermentation Production of L-lysine with Strain Being Introduced dapA* and lysC* (I)

The effect of the mutant DDPS on L-lysine production has been shown in Example 3. In order to achieve further improvement, the mutant AKIII gene obtained in Example 2 was allowed to co-exist with the mutant DDPS gene. The mutant AKIII gene to co-exist with the mutant DDPS gene was selected as originating from the No. 80 strain (lysC*80), judging from the enzyme activity, heat stability and the like.

lysC*80 was used after excising it from a plasmid pLLC*80 (FIG. 8) prepared by alternatively ligating lysC* having existed on pLYSC1*80 (hereinafter referred to as "lysC*80") at the downstream of a lacZ promoter of vector pHSG399 (produced by Takara Shuzo Co., Ltd.) which has an inverted-directional-insertion site with respect to pUC18 in order to increase the expression amount of lysC*. pLLC*80 is a plasmid prepared to arrange lysC*80 to allow the direction of transcription to have a normal orientation with respect to the lacZ promoter in order to improve the productivity of L-lysine because lysC*80 on pLYSC1*80 has its direction of transcription arranged in a reversed orientation with respect to the lacZ promoter.

A plasmid, RSFD80, having dapA* and lysC* was prepared from pLLC*80 and RSF24P obtained in Example 3 as shown in FIG. 9. RSFD80 includes dapA*24 and lysC*80 arranged in this order to allow the direction of transcription to have a normal orientation with respect to tetP at the downstream from a promoter (tetp) of a tetracycline resistance gene.

The RSFD80 µplasmid was introduced into an *E. coli* JM109 strain, which was designated as AJ12396. AJ12396 is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology on Oct. 28, 1993, as accession number of FERM P-13936, and transferred from the original deposition to international deposition based on Budapest Treaty on Nov. 1, 1994, and has been deposited as accession number of FERM BP-4859.

Strains harboring pLYSC1*24, pLYSC1*43, pLYSC1*48, pLYSC1*60, pLYSC1*117, pLYSC1*126, pLYSC1*149, pLYSC1*150, pLYSC1*156, pLYSC1*158, pLYSC1*167, pLYSC1*169 and pLYSC1*172 were not deposited. However, all of the mutation points of *lysC** on each of the plasmids have been clarified as described above. Thus it is easy for those skilled in the art that the plasmid is recovered from the aforementioned deposited bacterium by using a method of Maniatis et al. (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1.21 (1989)), and a target gene is obtained by using a site-directed mutagenesis method (Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 15.63 (1989)). RSFD80 was introduced into B-399 strain in accordance with an ordinary method, and B-399/RSFD80 was obtained. The L-lysine productivity of B-399/RSFD80 was evaluated. The L-lysine productivity was also evaluated for B-399/RSFP as a control.

The cultivation was performed at an agitation of 114–116 rpm under a condition of a cultivation period of 48 hours and a temperature of 37° C. by using the same medium for production of L-lysine as in Example 3. Results are shown in Table 6.

TABLE 6

Bacterial strain	Production amount of L-lysine hydrochloride
B-399/RSFD80	9.2 g/L
B-399/RSFP	0 g/L

EXAMPLE 5

Fermentation Production of L-lysine with Strain Being Introduced *dapA** and *lysC** (II)

It has been confirmed in Example 4 that the productivity of L-lysine can be improved by allowing the bacterium belonging to the genus *Escherichia* to harbor the mutant *dapA* gene and the mutant *lysC* gene. Experiments were performed to confirm whether or not this effect was maintained when the host is changed.

An *E. coli* W3110(*tyrA*) strain was used as a host. The W3110(*tyrA*) strain is described in detail in European Patent Publication No. 488424/92. Its preparation method will be briefly described as follows. The *E. coli* W3110 strain was obtained from National Institute of Genetics (Mishima-shi, Shizuoka-ken, Japan). This strain was spread on an LB plate containing streptomycin, and a streptomycin resistant strain was obtained by selecting strains which formed colonies. The selected streptomycin resistant strain was mixed with an *E. coli* K-12 ME8424 strain, and stationarily cultivated in a complete medium (L-Broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl) under a condition of 37° C. for 15 minutes to induce conjugation. The *E. coli* K-12 ME8424 strain has genetic characters of (HfrPO₄₅, thi, relA1, *tyrA*::Tn10, ung-1, nadB), which is available from National Institute of Genetics.

The culture was then spread on a complete medium (L-Broth: 1% Bacto trypton, 0.5% Yeast extract, 0.5% NaCl, 1.5% agar) containing streptomycin, tetracycline and L-tyrosine, and a colony-forming strain was selected. This strain was designated as *E. coli* W3110(*tyrA*) strain.

By the way, European Patent Publication No. 488424/92 describes many strains formed by introducing plasmids into the W3110(*tyrA*) strain. For example, a strain obtained by introducing a plasmid pHATerm is designated as *E. coli*

W3110(*tyrA*)/pHATerm strain, and deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology, to which a registration No. of FERM BP-3653 is given. The W3110(*tyrA*) strain can be also obtained by curing the plasmid pHATerm from the *E. coli* W3110(*tyrA*)/pHATerm strain. The curing of the plasmid can be performed in accordance with an ordinary method.

The plasmid RSFD80 containing both of *dapA** and *lysC** obtained in Example 4 was introduced into the W3110(*tyrA*) obtained as described above, and W3110(*tyrA*)/RSFD80 was obtained. The L-lysine productivity was evaluated for W3110(*tyrA*)/RSFD80. As a control, RSFP was introduced into the W3110(*tyrA*) strain in accordance with an ordinary method, and W3110(*tyrA*)/RSFP was obtained. The L-lysine productivity was also evaluated for W3110(*tyrA*)/RSFP as a control.

The cultivation was performed at an agitation of 114–116 rpm under a condition of a cultivation period of 48 hours and a temperature of 37° C. by using the aforementioned medium for L-lysine production. Results are shown in Table 7.

TABLE 7

Bacterial strain	Production amount of L-lysine hydrochloride
W3110 (<i>tyrA</i>) /RSFD80	8.9 g/L
W3110 (<i>tyrA</i>) /RSFP	0 g/L

EXAMPLE 6

Analysis of Rate Determining Steps of L-lysine Biosynthesis System and Improvement in L-lysine Productivity of L-lysine-producing Bacteria Belonging to the Genus *Escherichia*

It was tried to improve the L-lysine productivity by analyzing rate determining steps of the L-lysine biosynthesis system of *E. coli* and enhancing genes for enzymes which catalyze the steps.

<1> Identification of the First Rate Determining Steps (6-1-1) Preparation of Genes of L-lysine Biosynthesis System

The rate determining step was identified by isolating various genes of the L-lysine biosynthesis system, introducing these genes into *E. coli*, and examining effects of each of the genes on the L-lysine productivity. The introduced genes for enzymes of the L-lysine biosynthesis system, and the enzymes encoded by them are as follows.

ppc: phosphoenolpyruvate carboxylase
 aspc: aspartate aminotransferase
 lysC: aspartokinase III
 lysC*80: inhibition-desensitized aspartokinase III
 asd: aspartate semialdehyde dehydrogenase
 dapA: dihydrodipicolinate synthase
 dapA*24: inhibition-desensitized dihydrodipicolinate synthase
 dapB: dihydrodipicolinate reductase
 DDH: diaminopimelate dehydrogenase (originating from *Brevibacterium lactofermentum*)
 lysA: diaminopimelate decarboxylase

The L-lysine biosynthesis system from phosphoenolpyruvic acid to L-lysine can be thoroughly covered by the genes described above. The *dapC*, *dapD*, *dapE* and *dapF* genes, among the genes of the L-lysine biosynthesis system origi-

nally possessed by *E. coli*, are replaced with the gene DDH coding for DDH (diaminopimelate dehydrogenase) of *Brevibacterium lactofermentum* which can catalyze reactions concerning these gene products by itself. The W3110(tyrA) strain of the *E. coli* K-12 series was used as a host for introducing these genes.

The dapA and dapA*24 genes were respectively obtained by excision from pdapA2 and pdapAS24 (see Example 1) with EcoRI and KpnI (FIG. 10). These genes were ligated with pMW118 which was digested with EcoRI and KpnI to obtain pdapA and pdapA*. The lysC and lysC*80 genes were respectively obtained by excision from pLYSC1 and pLLC*80 (see Example 2) with EcoRI and SphI. These genes were ligated with pMW119 which was digested with EcoRI and SphI to obtain plysC and plysC* (FIG. 11).

The ppc gene was obtained from a plasmid pT2 having this gene. pT2 was cut with SmaI and ScaI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pppc (FIG. 12). *E. coli* F15 (AJ12873) harboring pT2 is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under an accession number of FERM BP-4732.

The aspC gene was obtained from a plasmid pLF4 (Inokuchi, K. et al., *Nucleic Acids Res.*, 10, 6957 (1982)) having this gene (FIG. 13). pLF4 was cut with PvuII and StuI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW119 to obtain a plasmid paspc.

The asd gene was obtained from a plasmid pAD20 (Haziza, C. et al., *EMBO*, 1, 379 (1982)) having this gene. pAD20 was cut with AseI and ClaI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pasd (FIG. 14).

The dapB gene was obtained by amplifying a dapB gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:9, NO:10) prepared on the basis of a nucleotide sequence of a known dapB gene (Bouvier, J. et al., *J. Biol. Chem.*, 259, 14829 (1984)) (FIG. 15). An obtained amplified DNA fragment was cut with AseI and DraI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW119 to obtain a plasmid pdapB.

The DDH gene was obtained by amplifying a DDH gene from chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:11, NO:12) prepared on the basis of a known nucleotide sequence of a DDH gene of *Corynebacterium glutamicum* (Ishino, S. et al., *Nucleic Acids Res.*, 15, 3917 (1987)). An obtained amplified DNA fragment was cut with EcoT22I and AseI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW119 to obtain a plasmid pDDH (FIG. 16).

The lysA gene was obtained by amplifying a lysA gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:13, NO:14) prepared on the basis of a nucleotide sequence of a known lysA gene (Stragier, P. et al., *J. Mol. Biol.*, 168, 321 (1983)). An obtained amplified DNA fragment was cut with SphI and BclI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid plysA (FIG. 17).

Confirmation of the fact that each of the aforementioned genes was cloned was performed by cutting them with restriction enzymes shown in the figures. The vectors

pMW118 and pMW119 (produced by Nippon Gene) used for cloning of these genes were selected because they were able to co-exist in cells of *E. coli* together with RSF1010 as a vector used for preparation of plasmids for lysine production described below, and also had a stable distribution mechanism.

(6-1-2) L-lysine Productivity of *E. coli* with Introduced Genes of L-lysine Biosynthesis System

E. coli W3110(tyrA) was transformed with each of the plasmids containing the genes of the L-lysine biosynthesis system described above, and obtained transformants were cultivated to perform L-lysine production. The cultivation was performed for 30 hours under a condition of a cultivation temperature of 37° C. and an agitation of 114–116 rpm by using the following medium. Results are shown in Table 8.

(Medium Composition)

Glucose	40 g/l
MgSO ₄ •7H ₂ O	1 g/l
(NH ₄) ₂ SO ₄	16 g/l
KH ₂ PO ₄	1 g/l
FeSO ₄ •7H ₂ O	0.01 g/l
NaSO ₄ •5H ₂ O	0.01 g/l
Yeast Ext. (Difco)	2 g/l
L-tyrosine	0.1 g/l
pH was adjusted to 7.0 with KOH to be autoclaved at 115° C. for 10 minutes (Glucose and MgSO ₄ •7H ₂ O were separately sterilized).	
Pharmacopoeial CaCO ₃	25 g/l
(heat-sterilized in dry state at 180° C. for 2 days)	
Antibiotics	
(streptomycin 20 mg/l or ampicillin 50 mg/l depending on species of plasmids to be introduced)	

TABLE 8

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)	0.08	0.2
W3110(tyrA)/pppc	0.08	0.2
W3110(tyrA)/paspc	0.12	0.3
W3110(tyrA)/plysC	0.08	0.2
W3110(tyrA)/plysC*	2.27	5.57
W3110(tyrA)/pasd	0.12	0.3
W3110(tyrA)/pdapA	2.32	5.70
W3110(tyrA)/pdapA*	3.63	8.90
W3110(tyrA)/pdapB	0.08	0.2
W3110(tyrA)/pDDH	0.08	0.2
W3110(tyrA)/plysA	0.12	0.3

i *E. coli*W3110(tyrA) became to produce L-lysine by introduction of plysC*, pdapA or pdapA*. Since both of lysC product and dapA product suffer feedback inhibition by L-lysine, it can be postulated that these enzymes are major regulatory points in L-lysine biosynthesis. The reaction catalyzed by dapA product exists in a position of branching to a biosynthesis system for L-threonine, L-methionine and L-isoleucine and a biosynthesis system for L-lysine, and is the first step of the biosynthesis system inherent to L-lysine. It was already reported that *E. coli* also becomes to produce L-lysine by amplification of a wild type dapA (Eur. J. Appl. Microbiol. Biotechnol., 15, 227 (1982)), which has been also confirmed from the result described above. On the other hand, the result of Example 3 has been confirmed again in that the yield of L-lysine is further increased when dapA* as an inhibition-desensitized type gene is introduced into *E. coli*.

Crude enzyme solutions were prepared from W3110 (tyrA), W3110(tyrA)/pdapA and W3110(tyrA)/pdapA* in the same manner as in Example 1, the DDPS (dihydropicolinate synthase) activity was measured, and the degree of inhibition of the DDPS activity by L-lysine was examined. Results are shown in Table 9.

TABLE 9

Bacterial strain	Specific activity *1	Degree of desensitization of inhibition *2
W3110(tyrA)	0.0423	50
W3110(tyrA)/pdapA	0.2754	22.9
W3110(tyrA)/pdapA*	0.1440	76.5

*1: $\mu\text{mols/min/mg protein}$

*2: ratio of activity maintenance (%) in the presence of 5 mM of L-lysine

The inhibition-desensitized dapA* product probably has a large effect on L-lysine production because it has a high degree of desensitization of inhibition although it has a lower specific activity than the wild type enzyme (about 1/2). The necessity of the desensitization of inhibition of the dapA product has been shown for L-lysine production.

In addition, the fact that lysC* has an effect on L-lysine production can be considered as follows. The first rate determining step is a step at which HD (homoserine dehydrogenase: product of thrA or metLM) competes with DDPS (dapA product) in acquiring ASA (aspartate- β -semialdehyde) as a substrate to serve at a branching point of the biosynthesis system, and when dapA is enhanced as described above, the reaction flows in a direction of L-lysine biosynthesis. On the other hand, it is speculated that when the supply amount of ASA is increased by enhancing lysC which participates in a reaction further upstream from dapA, any of reactions relevant to HD and DDPS is also facilitated, and thus the production amount of L-lysine has been also increased. However, this effect is scarcely obtained by enhancement of the wild type lysC only. This is probably because the inhibition of the wild type AKIII (lysC product) by L-lysine is more strict than that of the wild type DDPS (AKIII and DDPS are inhibited by about 100% and 80% respectively in the presence of 5 mM of L-lysine).

According to the facts described above, it was judged that the reaction by DDPS having a higher lysine-producing effect was the first rate determining step, and it was postulated that the reaction by AKIII was the second rate determining step.

<2> Identification of the Second Rate Determining Step

The second rate determining step was identified by enhancing various genes of the L-lysine biosynthesis system in strains with introduced dapA*. In order that other plasmids were stably harbored when they were introduced into *E. coli* harboring a plasmid containing dapA*, dapA* was transferred from pdapA to RSF1010, and RSF24P was obtained (FIG. 7). *E. coli* W3110(tyrA) was transformed with the plasmid RSF24P having dapA*.

Plasmids having genes of the L-lysine biosynthesis system were introduced into *E. coli* W3110(tyrA)/RSF24P. Two species of plasmids, namely RSF24P and a plasmid containing another gene of the L-lysine biosynthesis system, co-exist in each of obtained transformants. The L-lysine productivity was examined for these strains in the same manner as in (6-1-2). Results are shown in Table 10.

TABLE 10

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/RSF24P	3.63	8.9
W3110(tyrA)/RSF24P + pppc	3.67	9.0
W3110(tyrA)/RSF24P + paspC	3.59	8.8
W3110(tyrA)/RSF24P + plysC	3.42	8.4
W3110(tyrA)/RSF24P + plysC*	9.17	22.5
W3110(tyrA)/RSF24P + pasd	3.75	9.2
W3110(tyrA)/RSF24P + pdapA	3.55	8.7
W3110(tyrA)/RSF24P + pdapA*	3.46	8.5
W3110(tyrA)/RSF24P + pdapB	4.08	10.0
W3110(tyrA)/RSF24P + pDDH	3.67	9.0
W3110(tyrA)/RSF24P + plysA	3.55	8.7

As a result, a remarkable enhancing effect on the L-lysine productivity was found only in lysC*. The wild type lysC had no effect at all. This is probably because the inhibition by L-lysine is strong as described above. Thus it was confirmed that the reaction participated by lysC* was the second rate determining step.

lysC* was integrated into RSF24P, and RSFD80 was obtained (FIG. 9). In the same manner, lysC was integrated into RSF24P, and an obtained plasmid was designated as RSFD1. These plasmids were introduced into *E. coli* W3110 (tyrA), crude enzyme solutions were prepared, and the AK activity and the degree of inhibition of AK activity by L-lysine were examined in the same manner as in (6-1-2). Results are shown in Table 11.

TABLE 11

Bacterial strain for AK activity	Specific activity *1	Degree of desensitization of inhibition *2
W3110(tyrA)/RSF24P	0.94	42.9
W3110(tyrA)/RSFD1	18.55	7.2
W3110(tyrA)/RSFD80	33.36	98.8

*1: $\text{nmols/min/mg protein}$

*2: ratio of activity maintenance (%) in the presence of 5 mM of L-lysine

The specific activities of AK of the strains harboring the plasmids were increased 20–30 times by integrating lysC and lysC* into RSF24P. *E. coli* has three species of AK's, and lysC codes for AKIII among them. However, a total activity of the three species of AK's was measured in the experiment described above. It is speculated that the inhibition by L-lysine also becomes high in the strain harboring RSFD1 with the inserted wild type lysC because the ratio occupied by AKIII is higher than those by AKI and AKII as compared with the control (W3110(tyrA)/RSF24P), resulting in no indication of the effect on enhancement of the L-lysine productivity. On the other hand, it was revealed that the inhibition was desensitized for about 100% of AKIII in the strain harboring RSFD80, and this fact contributed to the improvement in L-lysine production.

<3> Identification of the Third Rate Determining Step

Next, various plasmids of the L-lysine biosynthesis system were introduced into *E. coli* W3110(tyrA)/RSFD80, and cultivation for L-lysine production was performed. Cultivation results are shown in Table 12.

TABLE 12

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/RSFD80	9.17	22.5
W3110(tyrA)/RSFD80 + pppc	8.97	22.0
W3110(tyrA)/RSFD80 + paspC	9.05	22.2
W3110(tyrA)/RSFD80 + plysC	8.56	21.0
W3110(tyrA)/RSFD80 + plysC*	8.15	20.0
W3110(tyrA)/RSFD80 + pasd	8.35	20.5
W3110(tyrA)/RSFD80 + pdapA	8.56	21.0
W3110(tyrA)/RSFD80 + pdapA*	8.15	20.0
W3110(tyrA)/RSFD80 + pdapB	10.80	26.5
W3110(tyrA)/RSFD80 + pDDH	8.56	21.0
W3110(tyrA)/RSFD80 + plysA	8.48	20.8

An enhancing effect on the L-lysine productivity was observed only in dapB, and it was found that the reaction participated by dapB was the third rate determining step. Thus dapB was inserted into RSFD80, and pCAB1 was obtained (FIG. 18). This plasmid was introduced into *E. coli* W3110(tyrA), a crude enzyme solution was prepared, and the enzyme activity of DDPR (dihydrodipicolinate reductase) was measured in accordance with a method described by Tamir, H. and Gilvarg, C., *J. Biol. Chem.*, 249, 3034 (1974). In the same manner, crude enzyme solutions were prepared from a strain harboring only RSFD80 and a strain harboring both RSFD80 and pdapB, and the DDPR activity was measured. Results are shown in Table 13.

TABLE 13

Bacterial strain	Specific activity (μ mol/min/mg protein)
W3110(tyrA)/RSFD80	0.027
W3110(tyrA)/RSFD80 + pdapB	0.092
W3110(tyrA)/PCAB1	0.178

The DDPR activity was increased about 3 times in the strain harboring RSFD80 and pdapB, and it was increased about 6.5 times in the strain harboring pCAB1 in which dapB was inserted into RSFD80, as compared with the control (strain harboring RSFD80 only). According to the fact that both W3110(tyrA)/RSFD80+pdapB and W3110(tyrA)/pCAB1 had equivalent L-lysine accumulation of 10.8 g/l, it was judged that dapB was provided in a sufficient amount for L-lysine production, and the rate determining step was shifted to the next step.

<4> Identification of the Fourth Rate Determining Step

Next, the fourth rate determining step was identified by using the plasmid pCAB1 harboring lysC*, dapA* and dapB. Various plasmids of the L-lysine biosynthesis system were introduced into *E. coli* W3110(tyrA)/pCAB1, and cultivation for L-lysine production was performed. Cultivation results are shown in Table 14.

TABLE 14

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/pCAB1	10.80	26.5
W3110(tyrA)/pCAB1 + pppc	11.00	27.0
W3110(tyrA)/pCAB1 + paspC	10.88	26.7
W3110(tyrA)/pCAB1 + plysC	10.60	26.0
W3110(tyrA)/pCAB1 + plysC*	10.39	25.5
W3110(tyrA)/pCAB1 + pasd	10.19	25.0

TABLE 14-continued

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
W3110(tyrA)/pCAB1 + pdapA	10.72	26.3
W3110(tyrA)/pCAB1 + pdapA*	10.80	26.5
W3110(tyrA)/pCAB1 + pdapB	10.92	26.8
W3110(tyrA)/pCAB1 + pDDH	12.23	30.0
W3110(tyrA)/pCAB1 + plysA	10.60	26.0

An enhancing effect on the L-lysine productivity was observed only in DDH, and it was found that the reaction catalyzed by DDH was the fourth rate determining step. In addition, SDAP (N-succinyl-L-L- α , ϵ -diaminopimelic acid) detected in a culture broth of the DDH non-introduced strain was not detected in a culture broth of the DDH introduced strain. Detection of SDAP was performed by means of TLC development (composition of development solvent; methanol:water:10N HCl:pyridine =80:17.5:2.5:10) (Bouvier, J., Richaud, C., Higgins, W., Bogler, O. and Stragier, P., *J. Bacteriol.*, 174, 5265 (1992)). Further, the color of broth was brown in the case of the DDH non-introduced strain, but it was changed to yellow in the case of the DDH introduced strain. Thus DDH was integrated into pCAB1 to prepared a plasmid pCABD2 (FIG. 19), and the DDH activity of *E. coli* W3110(tyrA) transformed with this plasmid was measured. The DDH enzyme activity was measured in accordance with a literature (Azizono, Haruo, *Fermentation and Industry*, 45, 964 (1987)). Results are shown in Table 15.

TABLE 15

Bacterial strain	Specific activity (μ mol/min/mg protein)
W3110(tyrA)/pCAB1	0.000
W3110(tyrA)/pCAB1 + pDDH	0.799
W3110(tyrA)/pCABD2	2.214

The DDH activity was not detected in the control (W3110(tyrA)/pCAB1) because DDH was originally not present in *E. coli*. The specific activity of DDH of the strain harboring pCABD2 (W3110(tyrA)/pCABD2) was about 2.5 times that of the strain harboring pDDH (W3110(tyrA)/pCAB1+pDDH), however, the both strain had an equivalent L-lysine accumulation amount (12.23 g/l). Thus it was judged that the DDH expression amount of pCABD2 was a sufficient amount.

<5> Analysis of Rate Determining Steps Among dapC, dapD, dapE and dapF

Next, in order to examine a rate limiting order of dapC, dapD, dapE and dapF replaced by DDH in the analysis described above, at first these genes were cloned. dapC was not cloned because of no report on its base sequence, however, the remaining three species of genes were cloned in accordance with the PCR method.

The dapD gene was obtained by amplifying a dapD gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:15, NO:16) prepared on the basis of a nucleotide sequence of a known dapD gene (Richaud, C. et al., *J. Biol. Chem.*, 259, 14824 (1984)). An obtained amplified DNA fragment was cut with Eco0109I and SacI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pdapD (FIG. 20).

The dapE gene was obtained by amplifying a dapE gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:17, NO:18) prepared on the basis of a nucleotide sequence of a known dapE gene (Bouvier, J. et al., *J. Bacteriol.*, 174, 5265 (1992)). An obtained amplified DNA fragment was cut with MunI and BalII, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pdapE (FIG. 21).

The dapF gene was obtained by amplifying a dapF gene from chromosomal DNA of an *E. coli* W3110 strain by means of the PCR method by using two species of oligonucleotide primers (SEQ ID NO:19, NO:20) prepared on the basis of a nucleotide sequence of a known dapF gene (Richaud, C. et al., *Nucleic Acids Res.*, 16, 10367 (1988)). An obtained amplified DNA fragment was cut with PstI, and the termini were blunt-ended, followed by insertion into a SmaI site of pMW118 to obtain a plasmid pdapF (FIG. 22).

Each of the plasmids obtained as described above was introduced into W3110(tyrA)/pCAB1, and cultivation for L-lysine production was performed. In the previous experiment, the changes were observed in the color of broth and in the presence or absence of accumulation of the intermediate (SDAP) in addition to the L-lysine production amount between before and after the introduction of DDH. Thus the analysis of the rate determining step was performed also by using them as indexes. Results are shown in Table 16.

TABLE 16

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)	Color of broth	Accumulation of SDAP
W3110(tyrA)/pCAB1	10.80	26.5	brown	+
W3110(tyrA)/pCAB1 + pdapD	11.08	27.2	yellow	+
W3110(tyrA)/pCAB1 + pdapE	11.12	27.3	brown	-
W3110(tyrA)/pCAB1 + pdapF	10.96	26.9	brown	+
W3110(tyrA)/pCABD2	12.23	30.0	yellow	-

The production amount of L-lysine was increased a little by the enhancement of dapD or dapE, but DDH was not exceeded. Further, it was found that the change in color of broth and the accumulation of SDAP as an intermediate observed upon the introduction of DDH were independent phenomena with each other, the change in color of broth resulted from dapD, and the disappearance of SDAP resulted from dapE. The relation between dapE and SDAP may be postulated judging from the biosynthesis pathway of L-lysine. The enhancement of dapF had no effect on the improvement in L-lysine productivity.

dapE was excised from pdapE, and it was inserted into pdapD to prepare a plasmid pMWdapDE1 containing both dapE and dapD (FIG. 23). Further, a fragment containing dapE and dapD was excised from pMWdapDE1, and it was inserted into pCAB1 to prepare pCABDE1 (FIG. 24). Strains harboring pCAB1, pCABDE1 or pCABD2 and a strain harboring both pCABDE1 and pdapF were prepared, and cultivation for L-lysine production was performed by using these strains. Results are shown in FIG. 17.

TABLE 17

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)	Color of broth	Accumulation of SDAP
W3110(tyrA)/pCAB1	10.80	26.5	brown	+
W3110(tyrA)/pCABDE1	12.23	30.0	yellow	-
W3110(tyrA)/pCABDE1 + pdapF	11.82	29.0	yellow	-
W3110(tyrA)/pCABD2	12.23	30.0	yellow	-

It was found that the L-lysine production amount, the color of broth, and the presence or absence of accumulation of SDAP became equivalent to those in the case of the production of DDH by enhancing dapD and dapE in combination. In addition, it was found that further enhancement of dapF had no effect on the improvement in L-lysine productivity, and the reaction participated by dapF did not make rate limitation. The results described above can be interpreted as follows.

Upon the step of introduction of pCAB1, intermediates are accumulated at two steps of SKAP (N-succinyl- ϵ -keto-L- α -aminopimelic acid) and SDAP. Among these intermediates, SDAP was detected in an extracellular broth. Although SKAP was not detected, it was speculated to be accumulated in bacterial cells. The reason for such speculation resides in the color of broth. The color of broth is yellow in the case of the wild type strain (W3110(tyrA)) or the like producing no L-lysine. However, the broth becomes brown probably due to bacteriolysis or the like when a load is applied to growth. It is speculated that SDAP has a small load on growth because it is discharged to the outside of cells, and hence, the broth is improved to have a yellow color although the accumulation amount of SDAP increases when SKAP is metabolized by the enhancement of only dapD. However, even if dapD is enhanced, the accumulation amount of L-lysine does not increase unless rate limitation by further downstream dapE is desensitized.

<6> Conclusion

According to the results described above, it has been found that the L-lysine productivity is improved in a stepwise manner by performing (1) introduction of dapA*, (2) introduction of lysC*, (3) enhancement of dapB, and (4) enhancement of DDH or dapD and dapE in bacteria belonging to the genus *Escherichia*. Further, *E. coli*, in which the L-lysine productivity is improved in a stepwise manner, has been obtained.

<7> Analysis of Rate Determining Step of L-lysine Biosynthesis System in *E. coli* C Strain

In order to examine whether or not the conclusion obtained in the foregoing could be applied to strains other than the *E. coli* K-12 series, rate determining steps of an L-lysine biosynthesis system of an *E. coli* C strain (IFO 13891) were analyzed in the same manner as described above. The cultivation condition was the same as that of W3110 (tyrA), however, L-tyrosine was not added to the medium.

(1) Identification of the First Rate Determining Step

The *E. coli* C strain (IFO 13891) transformed with plasmids containing genes of the L-lysine biosynthesis system was cultivated in the medium for L-lysine production, and the production amount of L-lysine hydrochloride was measured. Results are shown in Table 18.

TABLE 18

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yields versus sugar (%)
C	0.08	0.2
C/pppc	0.08	0.2
C/paspC	0.12	0.3
C/plysC	0.08	0.2
C/plysC*	0.12	0.3
C/pasd	0.08	0.2
C/pdapA	0.32	0.8
C/pdapA*	0.71	1.75
C/pdapB	0.12	0.3
C/pDDH	0.08	0.2
C/plysA	0.08	0.2

In the same manner as in W3110 (tyrA), L-lysine was also accumulated in the medium by the C strain by introducing the wild type dapA and further the inhibition-desensitized type dapA*. lysC* had no effect on the L-lysine productivity.

(2) Identification of the Second Rate Determining Step p The plasmid RSF24P containing dapA* was introduced into the *E. coli* C strain, and plasmids containing genes of the L-lysine biosynthesis system were further introduced. Obtained transformants were cultivated in the medium for L-lysine production, and the production amount of L-lysine hydrochloride was measured. Results are shown in Table 19.

TABLE 19

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/RSF24P	0.71	1.75
C/RSF24P + pppc	0.71	1.74
C/RSF24P + paspC	0.69	1.70
C/RSF24P + plysC	0.65	1.60
C/RSF24P + plysC*	1.82	4.50
C/RSF24P + pasd	0.70	1.73
C/RSF24P + pdapA	0.71	1.75
C/RSF24P + pdapA*	0.69	1.70
C/RSF24P + pdapB	0.99	2.45
C/RSF24P + pDDH	0.73	1.80
C/RSF24P + plysA	0.69	1.70

It was found that lysC* had an effect on the improvement in L-lysine productivity even in the case of the C strain with transformed dapA*, and the reaction participated by lysC* was the second rate determining step.

(3) Identification of the Third Rate Determining Step

The plasmid RSFD80 containing dapA* and lysC* was introduced into the *E. coli* C strain, and plasmids containing genes of the L-lysine biosynthesis system were further introduced. Obtained transformants were cultivated in the medium for L-lysine production, and the production amount of L-lysine hydrochloride was measured. Results are shown in Table 20.

TABLE 20

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/RSFD80	1.82	4.5
C/RSFD80 + pppc	1.74	4.3
C/RSFD80 + paspC	1.82	4.5
C/RSFD80 + plysC	1.91	4.7

TABLE 20-continued

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/RSFD80 + plysC*	1.74	4.3
C/RSFD80 + pasd	1.82	4.5
C/RSFD80 + pdapA	1.95	4.8
C/RSFD80 + pdapA*	1.91	4.7
C/RSFD80 + pdapB	2.31	5.7
C/RSFD80 + pDDH	2.15	5.3
C/RSFD80 + plysA	1.95	4.8

In the same manner as in the W3110 strain, only dapB had an effect on the improvement in L-lysine productivity, and it was found to be the third rate determining step.

(4) Identification of the Fourth Rate Determining Step

The plasmid pCAB1 containing dapA*, lysC* and dapB was introduced into the *E. coli* C strain, and plasmids containing genes of the L-lysine biosynthesis system were further introduced. Obtained transformants were cultivated in the L-lysine-producing medium, and the production amount of L-lysine hydrochloride was measured. Results are shown in Table 21.

TABLE 21

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)
C/pCAB1	2.31	5.7
C/pCAB1 + pppc	2.23	5.5
C/pCAB1 + paspC	2.35	5.8
C/pCAB1 + plysC	2.27	5.6
C/pCAB1 + plysC*	2.19	5.4
C/pCAB1 + pasd	2.23	5.5
C/pCAB1 + pdapA	2.31	5.7
C/pCAB1 + pdapA*	2.27	5.6
C/pCAB1 + pdapB	2.23	5.5
C/pCAB1 + pDDH	2.59	6.4
C/pCAB1 + plysA	2.19	5.4

In the same manner as in the W3110 strain, only DDH had an effect on the improvement in L-lysine productivity, and it was found to be the fourth rate determining step.

(5) Analysis of Rate Determining Steps Among dapC, dapD, dapE and dapF

Plasmid harboring the dapD, dapE or dapF genes were introduced, instead of DDH, into the *E. coli* C strain harboring pCAB1, and cultivation for L-lysine production was performed. Results are shown in Table 22.

TABLE 22

Bacterial strain	Production amount of L-lysine hydrochloride (g/l)	Yield versus sugar (%)	Color of broth	Accumulation of SDAP
C/pCAB1	2.31	5.7	brown	+
C/pCAB1 + pdapD	2.43	6.0	yellow	+
C/pCAB1 + pdapE	2.35	5.8	brown	-
C/pCAB1 + pdapF	2.23	5.5	brown	+
C/pCABDE1	2.59	6.4	yellow	-
C/pCABDE1 + pdapF	2.43	6.0	yellow	-
C/pCABD2	2.59	6.4	yellow	-

It was found that the two steps of dapD and dapE also concerned the rate determining in the C strain in the same manner as in the W3110 strain.

As described above, the strains of K-12 and C belonging to the different series had the same rate determining order.

Thus it is believed that the entire species of *E. coli* can be applied with the concept that the L-lysine productivity can be improved in a stepwise manner by performing introduction of dapA* and lysC* and enhancement of dapB and DDH (or dapD and dapE) in this order.

Industrial Applicability

According to the present invention, there has been obtained a DDPS mutant gene originating from a bacterium belonging to the genus *Escherichia* in which feedback

inhibition by L-lysine is sufficiently desensitized. An L-lysine-producing bacterium more improved than those in the prior art has been able to be obtained by introducing the gene into a bacterium belonging to the genus *Escherichia* 5 harboring an aspartokinase in which feedback inhibition by L-lysine is desensitized.

Further, the L-lysine productivity can be improved in a stepwise manner by enhancing dapB and DDH (or dapD and dapE) of the aforementioned L-lysine-producing bacterium in this order.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 20

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCGCAACTAC TGACATGACG

20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTAAGCCAT CAAATCTCCC

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1197 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *ESCHERICHIA COLI*
- (B) STRAIN: MC1061

(ix) FEATURE:

- (A) NAME/KEY: prim_transcript
- (B) LOCATION: 248
- (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 272..1150
- (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

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 (B) LOCATION: 27..46
 (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(ix) FEATURE:

(A) NAME/KEY: primer_bind
 (B) LOCATION: 1156..1175
 (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(ix) FEATURE:

(A) NAME/KEY: RBS
 (B) LOCATION: 261..265
 (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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TGCATACAAC AATCAGAACG GTTCTGTCTG CTGTGCTTTA ATGCCATACC AAACGTACCA    240
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His Ala Asp Val Val Met Met Thr Leu Asp Leu Ala Asp Gly Arg Ile
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Ile Asn Gln Arg Leu Met Pro Leu His Asn Lys Leu Phe Val Glu Pro	
235 240 245	
AAT CCA ATC CCG GTG AAA TGG GCA TGT AAG GAA CTG GGT CTT GTG GCG	1060
Asn Pro Ile Pro Val Lys Trp Ala Cys Lys Glu Leu Gly Leu Val Ala	
250 255 260	
ACC GAT ACG CTG CGC CTG CCA ATG ACA CCA ATC ACC GAC AGT GGT CGT	1108
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GAG ACG GTC AGA GCG GCG CTT AAG CAT GCC GGT TTG CTG TAA	1150
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 292 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Ala Ser Gly Thr Ser Ala Ile Val Ser Val Gly Thr Thr Gly Glu Ser	
35 40 45	
Ala Thr Leu Asn His Asp Glu His Ala Asp Val Val Met Met Thr Leu	
50 55 60	
Asp Leu Ala Asp Gly Arg Ile Pro Val Ile Ala Gly Thr Gly Ala Asn	
65 70 75 80	
Ala Thr Ala Glu Ala Ile Ser Leu Thr Gln Arg Phe Asn Asp Ser Gly	
85 90 95	
Ile Val Gly Cys Leu Thr Val Thr Pro Tyr Tyr Asn Arg Pro Ser Gln	
100 105 110	
Glu Gly Leu Tyr Gln His Phe Lys Ala Ile Ala Glu His Thr Asp Leu	
115 120 125	
Pro Gln Ile Leu Tyr Asn Val Pro Ser Arg Thr Gly Cys Asp Leu Leu	
130 135 140	
Pro Glu Thr Val Gly Arg Leu Ala Lys Val Lys Asn Ile Ile Gly Ile	
145 150 155 160	
Lys Glu Ala Thr Gly Asn Leu Thr Arg Val Asn Gln Ile Lys Glu Leu	
165 170 175	
Val Ser Asp Asp Phe Val Leu Leu Ser Gly Asp Asp Ala Ser Ala Leu	
180 185 190	
Asp Phe Met Gln Leu Gly Gly His Gly Val Ile Ser Val Thr Thr Asn	
195 200 205	
Val Ala Ala Arg Asp Met Ala Gln Met Cys Lys Leu Ala Ala Glu Glu	
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His Phe Ala Glu Ala Arg Val Ile Asn Gln Arg Leu Met Pro Leu His	
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(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "SYNTHETIC DNA"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "SYNTHETIC DNA"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2147 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: ESCHERICHIA COLI
 - (B) STRAIN: MC1061
- (ix) FEATURE:
 - (A) NAME/KEY: -35_signal
 - (B) LOCATION: 242..249
 - (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"
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 - (B) LOCATION: 265..273
 - (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: S"
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 - (B) LOCATION: 536..555
 - (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"
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 - (B) LOCATION: 575..578
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  (B) LOCATION: 2128..2147
  (D) OTHER INFORMATION: /note= "IDENTIFICATION METHOD: E"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GTC GCG TTG ATT GGC AAT GAC CTG TCA AAA GCC TGC GGC GTT GGC AAA Val Ala Leu Ile Gly Asn Asp Leu Ser Lys Ala Cys Gly Val Gly Lys 390 395 400	1795
GAG GTA TTC GGC GTA CTG GAA CCG TTC AAC ATT CGC ATG ATT TGT TAT Glu Val Phe Gly Val Leu Glu Pro Phe Asn Ile Arg Met Ile Cys Tyr 405 410 415 420	1843
GGC GCA TCC AGC CAT AAC CTG TGC TTC CTG GTG CCC GGC GAA GAT GCC Gly Ala Ser Ser His Asn Leu Cys Phe Leu Val Pro Gly Glu Asp Ala 425 430 435	1891
GAG CAG GTG GTG CAA AAA CTG CAT AGT AAT TTG TTT GAG TAA Glu Gln Val Val Gln Lys Leu His Ser Asn Leu Phe Glu * 440 445 450	1933
ATACTGTATG GCCTGAAGC TATATTTCCG GCCGTATTGA TTTTCTTGTC ACTATGCTCA	1993
TCAATAAACG AGCCTGTACT CTGTTAACCA CGCTCTTTAT CGGAGAATAA TTGCCTTTAA	2053
TTTTTTTATC TGCATCTCTA ATTAATTATC GAAAGAGATA AATAGTTAAG AGAAGGCAAA	2113

-continued

ATGAATATTA TCAGTTCTGC TCGCAAGGA ATTC

2147

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 449 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Ser Glu Ile Val Val Ser Lys Phe Gly Gly Thr Ser Val Ala Asp
 1 5 10 15

Phe Asp Ala Met Asn Arg Ser Ala Asp Ile Val Leu Ser Asp Ala Asn
 20 25 30

Val Arg Leu Val Val Leu Ser Ala Ser Ala Gly Ile Thr Asn Leu Leu
 35 40 45

Val Ala Leu Ala Glu Gly Leu Glu Pro Gly Glu Arg Phe Glu Lys Leu
 50 55 60

Asp Ala Ile Arg Asn Ile Gln Phe Ala Ile Leu Glu Arg Leu Arg Tyr
 65 70 75 80

Pro Asn Val Ile Arg Glu Glu Ile Glu Arg Leu Leu Glu Asn Ile Thr
 85 90 95

Val Leu Ala Glu Ala Ala Ala Leu Ala Thr Ser Pro Ala Leu Thr Asp
 100 105 110

Glu Leu Val Ser His Gly Glu Leu Met Ser Thr Leu Leu Phe Val Glu
 115 120 125

Ile Leu Arg Glu Arg Asp Val Gln Ala Gln Trp Phe Asp Val Arg Lys
 130 135 140

Val Met Arg Thr Asn Asp Arg Phe Gly Arg Ala Glu Pro Asp Ile Ala
 145 150 155 160

Ala Leu Ala Glu Leu Ala Ala Leu Gln Leu Leu Pro Arg Leu Asn Glu
 165 170 175

Gly Leu Val Ile Thr Gln Gly Phe Ile Gly Ser Glu Asn Lys Gly Arg
 180 185 190

Thr Thr Thr Leu Gly Arg Gly Gly Ser Asp Tyr Thr Ala Ala Leu Leu
 195 200 205

Ala Glu Ala Leu His Ala Ser Arg Val Asp Ile Trp Thr Asp Val Pro
 210 215 220

Gly Ile Tyr Thr Thr Asp Pro Arg Val Val Ser Ala Ala Lys Arg Ile
 225 230 235 240

Asp Glu Ile Ala Phe Ala Glu Ala Ala Glu Met Ala Thr Phe Gly Ala
 245 250 255

Lys Val Leu His Pro Ala Thr Leu Leu Pro Ala Val Arg Ser Asp Ile
 260 265 270

Pro Val Phe Val Gly Ser Ser Lys Asp Pro Arg Ala Gly Gly Thr Leu
 275 280 285

Val Cys Asn Lys Thr Glu Asn Pro Pro Leu Phe Arg Ala Leu Ala Leu
 290 295 300

Arg Arg Asn Gln Thr Leu Leu Thr Leu His Ser Leu Asn Met Leu His
 305 310 315 320

Ser Arg Gly Phe Leu Ala Glu Val Phe Gly Ile Leu Ala Arg His Asn
 325 330 335

Ile Ser Val Asp Leu Ile Thr Thr Ser Glu Val Ser Val Ala Leu Thr
 340 345 350

-continued

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGCCCTCGA GCTAAATTAG 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGCACGGTAG GATGTAATCG 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTAATGAAAC AAATGCCCGG 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTTATTTCATA ATTGCCACCG 20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CACGGTAATA CATATAACCG 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCTGCAATTG TCAAACGTCC 20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCGACGCGC TTGAGATCTT 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCATAAAGAG TCGCTAAACG 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "SYNTHETIC DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAACCGCCCG GTCATCAAGC 20

What is claimed is:

1. An isolated DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia*, wherein the dihydrodipicolinate synthase has a mutation which desensitizes feedback inhibition by L-lysine, wherein the mutation is selected from the group consisting of
 - (a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,
 - (b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and
 - (c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,
 - (d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,
 - (e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and
 - (f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and

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synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue.

2. The isolated DNA of claim 1, wherein the mutation to desensitize feedback inhibition by L-lysine is selected from the group consisting of

- (a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,
- (b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and
- (c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the 118th histidine residue as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue,
- (d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,
- (e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and
- (f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the histidine residue corresponding to the 118th residue as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue.

3. A bacterium belonging to the genus *Escherichia* which is transformed with a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia* and having mutation to desensitize feedback inhibition by L-lysine, wherein the mutation is selected from the group consisting of

- (a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,
- (b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and
- (c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,
- (d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal

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in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue,

- (e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue, and
- (f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue and replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with another amino acid residue.

4. The bacterium of claim 3, wherein the mutation is selected from the group consisting of

- (a) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,
- (b) a mutation to replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and
- (c) a mutation to replace the alanine residue at the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the histidine residue at the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue,
- (d) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue,
- (e) a mutation to replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue, and
- (f) a mutation to replace the alanine residue corresponding to the 81st position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a valine residue and replace the histidine residue corresponding to the 118th position as counted from the N-terminal in the amino acid sequence of the dihydrodipicolinate synthase of SEQ ID NO: 4 with a tyrosine residue.

5. The bacterium of claim 3, further harboring an aspartokinase which is desensitized to feedback inhibition by L-lysine.

6. The bacterium of claim 5, which is obtained by introducing, into its cells, a DNA coding for an aspartokinase III originating from a bacterium belonging to the genus *Escherichia*, wherein the aspartokinase III has a mutation which desensitizes feedback inhibition by L-lysine.

7. The bacterium of claim 6, wherein the mutation is selected from the group consisting of

- (a) a mutation to replace the glycine residue at the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,

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- (n) a mutation to replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the glycine residue corresponding to the 408th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (o) a mutation to replace the glycine residue corresponding to the 34th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the glycine residue corresponding to the 323rd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (p) a mutation to replace the leucine residue corresponding to the 325th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (q) a mutation to replace the methionine residue corresponding to the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (r) a mutation to replace the methionine residue corresponding to the 318th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the valine residue corresponding to the 349th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (s) a mutation to replace the serine residue corresponding to the 345th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (t) a mutation to replace the valine residue corresponding to the 347th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (u) a mutation to replace the threonine residue corresponding to the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (v) a mutation to replace the threonine residue corresponding to the 352nd position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue and replace the serine residue corresponding to the 369th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue,
- (w) a mutation to replace the glutamic acid residue corresponding to the 164th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with another amino acid residue, and
- (x) a mutation to replace the methionine residue corresponding to the 417th position as counted from the

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- tokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the serine residue corresponding to the 369th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a phenylalanine residue,
- (w) a mutation to replace the glutamic acid residue corresponding to the 164th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a lysine residue, and
- (x) a mutation to replace the methionine residue corresponding to the 417th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with an isoleucine residue and replace the cysteine residue corresponding to the 419th position as counted from the N-terminal in the amino acid sequence of the aspartokinase III of SEQ ID NO: 8 with a tyrosine residue.
9. The bacterium of claim 5, wherein a dihydrodipicolinate reductase gene is enhanced.
10. The bacterium of claim 9, transformed with a recombinant DNA constructed by ligating the dihydrodipicolinate reductase gene with a vector autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.
11. The bacterium of claim 9, into which an enhanced diaminopimelate dehydrogenase gene originating from coryneform bacterium has been introduced.
12. The of claim 11, transformed with a recombinant DNA constructed by ligating the diaminopimelate dehydrogenase gene originating from a coryneform bacterium with a vector autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.
13. The bacterium of claim 9, wherein a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate deacylase gene are enhanced.
14. The bacterium of claim 13, transformed with a single recombinant DNA or two recombinant DNA's constructed by ligating the succinyldiaminopimelate transaminase gene and the succinyldiaminopimelate deacylase gene with an identical vector or different vectors autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.

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15. A method of producing L-lysine, comprising: cultivating the bacterium of claim 3 in a suitable culture medium, producing and accumulating L-lysine in the culture thereof, and collecting L-lysine from the culture.
16. A bacterium belonging the genus *Escherichia* which is transformed with a DNA coding for a dihydrodipicolinate synthase originating from a bacterium belonging to the genus *Escherichia* and having mutation to desensitize feedback inhibition by L-lysine, and further harboring an aspartokinase which is desensitized to feedback inhibition by L-lysine, and wherein a dihydrodipicolinate reductase gene is enhanced.
17. The bacterium of claim 16, transformed with a recombinant DNA constructed by ligating the dihydrodipicolinate reductase gene with a vector autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.
18. The bacterium of claim 16, into which an enhanced diaminopimelate dehydrogenase gene originating from coryneform bacterium has been introduced.
19. The of claim 18, transformed with a recombinant DNA constructed by ligating the diaminopimelate dehydrogenase gene originating from a coryneform bacterium with a vector autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.
20. The bacterium of claim 16, wherein a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate transaminase gene and a succinyldiaminopimelate deacylase gene are enhanced.
21. The bacterium of claim 20, transformed with a single recombinant DNA or two recombinant DNA's constructed by ligating the succinyldiaminopimelate transaminase gene and the succinyldiaminopimelate deacylase gene with an identical vector or different vectors autonomously replicable in cells of bacteria belonging to the genus *Escherichia*.
22. A method of producing L-lysine, comprising: cultivating the bacterium of claim 16 in a suitable culture medium, producing and accumulating L-lysine in the culture thereof, and collecting L-lysine from the culture.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,040,160
DATED : March 21, 2000
INVENTOR(S) : Kojima et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 67.

Line 34-35, "succinyldiaminopimelate transaminase" should read

-- tetrahydrodipicolinate succinylase --.

Lines 35-36, delete "and a succinyldiaminopimelate transaminase gene".

Line 40, "succinyldiaminopimelate transaminase" should read

-- tetrahydrodipicolinate succinylase --.

Column 68.

Line 3, "producing and accumulating" should read -- to produce and accumulate --.

Lines 26-27, "succinyldiaminopimelate transaminase" should read

-- tetrahydrodipicolinate succinylase --.

Lines 27-28, delete "and a succinyldiaminopimelate transaminase gene".

Line 32, "succinyldiaminopimelate transaminase" should read

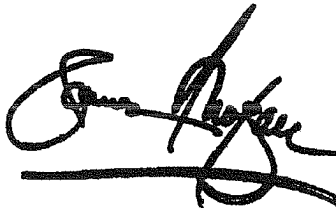
-- tetrahydrodipicolinate succinylase --.

Line 38, "producing and accumulating" should read -- to produce and accumulate --.

Signed and Sealed this

Second Day of July, 2002

Attest:

A handwritten signature in black ink, appearing to read "James E. Rogan", written over a horizontal line.

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office